

Nuclear Dbf2-related protein kinases (NDRs) in isolated cardiac myocytes and the myocardium: activation by cellular stresses and by phosphoprotein serine-/threonine-phosphatase inhibitors

Article

Accepted Version

Fuller, S. J., Pikkarainen, S., Tham, E. L., Cullingford, T. E., Molkentin, J. D., Cornils, H., Hergovitch, A., Hemmings, B. A., Clerk, A. and Sugden, P. H. (2008) Nuclear Dbf2-related protein kinases (NDRs) in isolated cardiac myocytes and the myocardium: activation by cellular stresses and by phosphoprotein serine-/threonine-phosphatase inhibitors. *Cellular Signalling*, 20 (8). pp. 1564-1577. ISSN 0898-6568 doi: <https://doi.org/10.1016/j.cellsig.2008.04.013> Available at <http://centaur.reading.ac.uk/18418/>

It is advisable to refer to the publisher's version if you intend to cite from the work. See [Guidance on citing](#).

To link to this article DOI: <http://dx.doi.org/10.1016/j.cellsig.2008.04.013>

Publisher: Elsevier

All outputs in CentAUR are protected by Intellectual Property Rights law, including copyright law. Copyright and IPR is retained by the creators or other copyright holders. Terms and conditions for use of this material are defined in the [End User Agreement](#).

www.reading.ac.uk/centaur

CentAUR

Central Archive at the University of Reading

Reading's research outputs online

1 **Nuclear Dbf2-related protein kinases (NDRs) in isolated cardiac**
2
3
4 **myocytes and the myocardium: Activation by cellular stresses and**
5
6 **by phosphoprotein serine-/threonine-phosphatase inhibitors**
7
8
9

10
11
12 Stephen J. Fuller^a Sampsa Pikkarainen^a, El Li Tham^a, Timothy E. Cullingford^a,
13
14 Jeffery D. Molkenin^b, Hauke Cornils^c, Alexander Hergovich^c, Brian A.
15 Hemmings^c, Angela Clerk^a, and Peter H Sugden^{a*}
16
17

18 ^a *National Heart and Lung Institute (NHLI) Division, Faculty of Medicine, Imperial College*
19 *London, Flowers Building, Armstrong Road, London SW7 2AZ, UK*
20
21

22 ^b *Department of Pediatrics, University of Cincinnati, Cincinnati Children's Hospital Medical*
23 *Center, 3333 Burnet Ave., Cincinnati, Ohio 45229-3039, USA*
24
25

26 ^c *Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH 4002 Basel,*
27 *Switzerland*
28
29
30

31 Running title: Nuclear Dbf2-related protein kinases in the heart
32
33
34
35
36
37

38 * Corresponding author. NHLI Division, Faculty of Medicine, Imperial College London,
39 Flowers Building (4th Floor), Armstrong Rd., London SW7 2AZ, UK. Tel.: +44 20 7594
40 3410; fax: +44 20 7594 3419.
41
42

43 *E-mail address:* p.sugden@imperial.ac.uk (P.H. Sugden).
44
45
46
47
48
49
50

Abstract

1
2
3
4
5 The nuclear Dbf2-related protein kinases 1 and 2 (NDR1/2) are closely-related AGC
6 family kinases that are strongly conserved through evolution. In mammals, they are activated
7
8 *inter alia* by phosphorylation of an hydrophobic domain threonine-residue [NDR1(Thr-
9
10 444)/NDR2(Thr-442)] by an extrinsic protein kinase followed by autophosphorylation of a
11
12 catalytic domain serine-residue [NDR1(Ser-281)/NDR2(Ser-282)]. We examined NDR1/2
13
14 expression and regulation in primary cultures of neonatal rat cardiac myocytes and in
15
16 perfused adult rat hearts. In myocytes, transcripts for NDR2, but not NDR1, were induced by
17
18 the hypertrophic agonist, endothelin-1. NDR1(Thr-444) and NDR2(Thr-442) were rapidly
19
20 phosphorylated (maximal in 15 - 30 min) in myocytes exposed to some phosphoprotein Ser-
21
22 /Thr-phosphatase 1/2 inhibitors (calyculin A, okadaic acid) and, to a lesser extent, by
23
24 hyperosmotic shock, low concentrations of H₂O₂, or chelerythrine. In myocytes adenovirally-
25
26 transduced to express FLAG-NDR2 (which exhibited a mainly-cytoplasmic localisation), the
27
28 same agents increased FLAG-NDR2 activity as assessed by *in vitro* protein kinase assays,
29
30 indicative of FLAG-NDR2(Ser-282/Thr-442) phosphorylation. Calyculin A-induced
31
32 phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 were
33
34 inhibited by staurosporine, but not by other protein kinase inhibitors tested. In *ex vivo* rat
35
36 hearts, NDR1(Thr-444)/NDR2(Thr-442) were phosphorylated in response to ischaemia-
37
38 reperfusion or calyculin A. From a pathological viewpoint, we conclude that activities of
39
40 NDR1 and NDR2 are responsive to cytotoxic stresses in heart preparations and this may
41
42 represent a previously-unidentified response to myocardial ischaemia *in vivo*.
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Keywords: Intracellular signalling; Protein phosphorylation; Nuclear Dbf2-related protein
kinases (NDRs); Heart; Phosphoprotein Ser-/Thr-phosphatase inhibitors; Cellular stresses;
Ischaemia and reperfusion injury

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1. Introduction

The nuclear Dbf2-related protein kinases (NDRs) are members of the AGC protein serine-/threonine-kinase family and are strongly conserved between species as diverse as yeasts and *Homo sapiens* [1,2]. Human (and mouse) cells express two isoforms encoded by separate genes, namely NDR1 (or serine-/threonine kinase 38, STK38) and NDR2 (or serine-/threonine kinase 38-like, STK38L) [1,2]. These cells also express two NDR1-/NDR2-related protein kinases, large tumour suppressor-1 (LATS1) and LATS2 [1,2]. The NDRs are widely-expressed in mouse [3] and human [4,5] tissues. In mouse, NDR1 transcripts are most-highly expressed in spleen and are also moderately expressed in brain, lung, thymus, testis and adipose tissue whereas NDR2 transcripts are most-highly expressed in the gastrointestinal tract (large and small intestine, stomach) and moderately expressed in testis [3]. The heart also expresses NDR1 and NDR2 transcripts, albeit at lower levels [3]. In human tissues, NDR1 and NDR2 are most highly expressed in thymus [4]. NDR1 is also highly expressed in human skeletal muscles whereas NDR2 is highly expressed in heart and brain [4]. It is not known whether these apparent differences in expression profiles between mouse and *Homo sapiens* are real or reflect inter-laboratory variations. The consensus sequence phosphorylated by this group of kinases has been variously reported to be (K/R)XX(S/T) (human NDR1 [6]), RXXS (preferred to RXXT) (yeast Dbf2 [7]) or HX(R/H/K)XX(S/T) (human LATS1 [8]). The physiological functions of the NDRs in mammals are obscure. NDR2 may be involved in the organisation of the actin cytoskeleton in pheochromocytoma 12 (PC12) cells and its transcript is also rapidly but transiently induced in mouse amygdalae following Pavlovian fear conditioning training [9]. Recently,

1 NDR1/NDR2 have been implicated in centrosome duplication with overexpression inducing
2 centrosome overduplication [10].
3
4
5
6

7 In their inactive state, NDRs are autoinhibited by an insert between the catalytic
8 domain subdomains VII and VIII (the autoinhibitory sequence) that is rich in basic residues
9 [11] and was originally thought to represent a nuclear localisation signal [5]. Activation of
10 NDRs involves regulatory phosphorylations and/or interactions with other proteins. As with
11 many AGC kinases, NDRs are phosphorylated on two conserved residues, one in the C-
12 terminal hydrophobic domain (Thr-444 in human, mouse or rat NDR1, Thr-442 in human or
13 mouse NDR2) and one in the activation loop (or T loop) of the catalytic domain (Ser-281 in
14 human, mouse or rat NDR1, Ser-282 in human or mouse NDR2) [3,12]. One protein kinase
15 which phosphorylates NDR1(Thr-444)/NDR2(Thr-442) is the mammalian serine-/threonine-
16 STE20-like kinase 3 (MST3) [13] with dephosphorylation catalysed by phosphoprotein
17 serine-/threonine-phosphatase (PPP) PP2A [3,12], now known as PPP2. In contrast (and
18 unusually for AGC kinases), phosphorylation of NDR1(Ser-281)/NDR2(Ser-282) is thought
19 to represent an autophosphorylation [3,13,14]. In addition, activation may involve
20 interactions with other proteins. Initially, the S100 Ca²⁺ binding protein was thought to be of
21 major importance and to account for the Ca²⁺-sensitivity of NDR activation [3,6,14]. One
22 suggestion is that phosphorylation of NDR2(Thr-75) in a S100/Ca²⁺-dependent manner leads
23 to phosphorylation of NDR2(Thr-442) in the phospho-NDR2(Thr-75) [NDR2(*P*Thr-75)]
24 species by an unknown kinase [14,15]. The NDR2(Ser-281) residue in the NDR2(*P*Thr-
25 75/*P*Thr-442) species is then autophosphorylated leading to activation [14,15]. However, the
26 emphasis has now switched to the Mps-one binder (MOB) proteins which interact with basic
27 residues in the N-terminal regulatory domains of the NDRs [4,11]. It is not entirely clear how
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 MOB proteins activate NDRs. One view is that they bring NDRs to the plasma membrane for
2 interaction with the upstream kinases for the hydrophobic domain phosphorylation, thus
3
4 allowing enhanced autophosphorylation of the activation loop [16]. However, other
5
6 mechanisms proposed are the release of the inhibition imposed by the autoinhibitory
7
8 sequence [11] or, in yeast, perturbation and disruption of inhibitory self-associations [17].
9
10
11
12
13

14
15 Our interests in NDRs were stimulated by the observation that a microarray screen of
16
17 the changes in transcript abundances following exposure of neonatal rat cardiac (ventricular)
18
19 myocytes to the Gq protein-coupled receptor agonist, endothelin-1 (ET-1), revealed that
20
21 NDR2 transcript abundances were increased >2-fold after 4 h [18]. In these cells, which
22
23 become terminally differentiated during the perinatal period and are thus unable to undergo
24
25 complete cycles of cell division, ET-1 is a powerful stimulator of hypertrophic growth, and
26
27 this response has pathophysiological relevance [19-21]. These effects of ET-1 are brought
28
29 about by rapid (within minutes) but transient activation of intracellular signalling pathways,
30
31 particularly the extracellular signal-regulated kinase 1/2 (ERK1/2) cascade [22,23], and rapid
32
33 but phasic activation of successive waves of gene expression, the earliest of which involve
34
35 expression of immediate early genes [18]. Many of the early changes in gene expression are
36
37 sensitive to inhibition of the ERK1/2 cascade [18,24]. Ultimately, these early changes lead to
38
39 expression of genes associated with the established hypertrophic response [25]. Here, we
40
41 have characterised the acute activation of NDRs in cardiac myocytes and have found that,
42
43 whilst the expression of NDR2 was stimulated by ET-1, their activities were stimulated by
44
45 cytotoxic interventions and particularly by PPP inhibitors.
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

2. Materials and methods

2.1. Materials

With the exception of ET-1 (which was from Bachem.), biochemical reagents were from Sigma-Aldrich, Calbiochem or Alexis/Axxora, and laboratory chemicals were from VWR. Anti-NDR antibodies were raised to residues 59-86 of *Homo sapiens* NDR1 (for the measurement of the total pool) [16], and to a phosphopeptide (residues 436-450) encompassing NDR1(PThr-444) for measurement of the species phosphorylated in the hydrophobic domain [14]. These antibodies also recognise human NDR2 and NDR2(PThr-442), respectively [13]. Since rat NDR1 is identical to human NDR1 and rat NDR2 is identical to human NDR2 in these immunogen regions, (see Supplementary Material, Fig. 1), these antibodies should and do recognise rat NDR1 and rat NDR2. Rabbit polyclonal anti-OctA (FLAG[®]) Probe (D-8) (sc-807) and mouse monoclonal anti-OctA (FLAG[®]) Probe (H-6) (sc-7787) were from Santa Cruz Biotechnology. Secondary antibodies and fluorescent mounting medium were from Dako. [γ -³²P]ATP, rainbow molecular weight markers, streptavidin-Texas red and Hyperfilm MP were from GE Healthcare.

2.2. Primary culture of neonatal rat cardiac myocytes

Myocytes were dissociated from the ventricles of 1- to 3-day-old Sprague-Dawley rat hearts by an adaptation of the method of Iwaki *et al.* [26] as previously described [27]. Unless stated otherwise, cells were plated in gelatin-coated Primaria culture dishes (BD Biosciences) at a density of 2×10^6 cells/35 mm dish or 4×10^6 cells/60 mm for 18 h in 15%

1 (v/v) foetal calf serum. Unless experiments involved adenoviral infection, serum was
2 withdrawn for the 24 h before experimentation.
3
4
5
6

7 *2.3. Infections with adenoviral vectors*

8
9

10
11
12 Details of the construction of the adenoviral vectors (Adv) encoding rat N-terminally
13 FLAG-tagged wild-type NDR2 (FLAG-NDR2) and N-terminally FLAG-tagged NDR2-
14 PIFtide {rat NDR2(1-432), i.e. lacking the hydrophobic domain residues 433-464, with the
15 human PIFtide [3-phosphoinositide-dependent kinase 1 (PKD1) interacting fragment] from
16
17 human PIFtide [3-phosphoinositide-dependent kinase 1 (PKD1) interacting fragment] from
18
19 protein kinase N2 (PKN2, residues 969-983) spliced to the C-terminus, FLAG-NDR2-
20
21 PIFtide} are provided in the ‘Supplementary Material’ section. For Adv infections other than
22
23 those for immunofluorescence staining, serum was withdrawn from myocytes (2×10^6
24
25 cells/35 mm dish) for approximately 6 h before infection with Adv encoding FLAG-NDR2 or
26
27 FLAG-NDR2-PIFtide . In initial experiments, the multiplicities of infection were titrated so
28
29 that the relative levels of expression of FLAG-NDR2 and endogenous NDR1/NDR2 were
30
31 similar, as assessed by immunoblotting using the rabbit polyclonal anti-NDR1/NDR2
32
33 antibody. Likewise, the multiplicity of infection of Adv.FLAG-NDR2-PIFtide was adjusted
34
35 so that expression of FLAG-NDR2-PIFtide and FLAG-NDR2 was approximately equal as
36
37 shown using the rabbit polyclonal anti-FLAG antibody. Myocytes were used for
38
39 experimentation (no change of medium) at 20 h after they had been infected.
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

2.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Following exposure to agonists, myocytes were washed twice with ice-cold phosphate-buffered saline (PBS). For whole cell extracts, myocytes were scraped into 150 μ l/dish of Buffer A [20 mM β -glycerophosphate pH 7.5, 2 mM EDTA, 5 mM dithiothreitol (DTT), 50 mM NaF, 0.2 mM Na₃VO₄, 2 μ M microcystin LR, 10 mM benzamidine, 300 μ M phenylmethylsulphonyl fluoride (PMSF), 200 μ M leupeptin, 10 μ M *trans*-epoxy-succinyl-l-leucylamido-(4-guanidino)-butane (E64), containing 1% (v/v) Triton X-100]. Samples were incubated on ice for 10 min and then centrifuged (10,000 \times g, 5 min, 4°C). The supernatants were retained and protein contents measured by the Bradford assay (BioRad) [28]. Samples were boiled with 0.33 vol of SDS-PAGE sample buffer [10% SDS (w/v), 13% glycerol (v/v), 300 mM Tris-HCl pH 6.8, 130 mM DTT, 0.2% bromophenol blue (w/v)]. Proteins (20 – 50 μ g/lane, but equal for each blot) were separated by SDS-PAGE using 10% (w/v) resolving gels and 4% (w/v) stacking gels, and transferred to nitrocellulose as described previously [29]. Non-specific binding sites were blocked with 5% (w/v) non-fat milk powder in TBST [20 mM Tris-HCl pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween 20]. Membranes were incubated (overnight, 4°C) with primary antibodies [1:300 for total NDR1/NDR2 and for NDR1(*P*Thr-444)NDR2(*P*Thr-442)] diluted in TBST containing 5% (w/v) bovine serum albumin. The NDR1(*P*Thr-444)NDR2(*P*Thr-442) antibody in TBST/albumin could be used repeatedly providing it was stored at 4°C in the interim. Membranes were washed in TBST, incubated (60 min, room temperature) with horseradish peroxidase-conjugated secondary antibodies (1/5,000) in TBST containing 1% (w/v) nonfat milk powder, and were then washed in TBST. Bands were detected by enhanced

1 chemiluminescence (Santa Cruz Biotechnology) using Hyperfilm MP and were quantified by
2 scanning densitometry.
3
4
5
6

7 *2.5. Assay of FLAG-NDR2 activity (immunoprecipitation kinase assays)*
8
9

10
11
12 Myocytes were washed twice with ice-cold PBS and extracted into 100 μ l of Buffer B
13 [20 mM Tris-HCl pH 7.5, 10% (v/v) glycerol, 100 mM KCl, 5 mM MgCl₂, 1 mM EDTA,
14 0.05 % (v/v) 2-mercaptoethanol, 5 mM DTT, 5 mM NaF, 0.2 mM Na₃VO₄, 2 μ M microcystin
15 LR, 300 μ M PMSF, 200 μ M leupeptin, 10 μ M E64, containing 1% (v/v) Triton X-100].
16
17 Samples were incubated on ice for 10 min and then centrifuged (10,000 \times g, 5 min, 4°C).
18
19 The supernatants were retained and samples were taken for immunoprecipitation (75 or 90%
20 of the supernatant used), protein determination by Bradford assay [28] and occasionally for
21 immunoblotting. For immunoprecipitation, samples were added to Eppendorf tubes
22 containing 20 μ l of protein A-Sepharose (1:1 slurry in Buffer B) and 5 μ l of rabbit anti-FLAG
23 antibody that had been pre-incubated together overnight at 4°C with rotation. Samples were
24 rotated overnight at 4°C and the pellets collected by centrifugation (10,000 \times g, 1 min, 4°C).
25
26 The pellets were washed three times in Buffer B (0.7 ml), once with kinase assay buffer (150
27 μ l, 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1 μ M microcystin LR, 4 μ M leupeptin, 1 mM
28 benzamidine) and finally resuspended in 45 μ l of kinase assay buffer containing 1 mM
29 KKRNRRLSVA peptide substrate [6]. The recovery of FLAG-NDR2 or FLAG-NDR2-
30 PIFtide (estimated by immunoblotting using the monoclonal anti-FLAG antibody following
31 immunoprecipitation with the rabbit polyclonal anti-FLAG antibody) was about 75% of the
32 input.
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 FLAG-NDR2 or FLAG-NDR2-PIFtide assays were initiated by the addition of 5 μ l of
2 1 mM [γ - 32 P]ATP(1 μ Ci/assay)/100 mM MgCl₂ followed by incubation at 30°C for 30 min.
3
4 Reactions were terminated by the addition of 5 μ l 0.5 M EDTA, pH7.5, followed by cooling
5
6 on ice. Samples were centrifuged (10,000 \times g, 1 min, 4°C) and duplicate samples (20 μ l) of
7
8 the supernatants were spotted onto P81 paper squares which were then washed in 75 mM
9
10 H₃PO₄ (4 \times 15 min) on a rocking platform. Radioactivity was measured by Čerenkov
11
12 radiation counting. Blanks (one with kinase assay buffer + [γ - 32 P]ATP/MgCl₂ and one
13
14 involving assay of KKRNRRLSVA kinase activity with immunoprecipitates from cells
15
16 infected with ‘empty’ FLAG-tag Adv) were routinely included.
17
18
19
20
21
22
23
24

25 2.6. *Ex vivo heart preparations*

26
27
28
29
30 Hearts of female rats (279 \pm 6 g body wt.) that had littered 7-10 days previously were
31
32 perfused retrogradely at 10 kPa with Krebs-Henseleit bicarbonate-buffered saline as described
33
34 previously [30]. In each set of experiments, following a 15 min retrograde stabilisation
35
36 perfusion, hearts were either (i) perfused retrogradely for a further 20 min, (ii) rendered
37
38 globally-ischemic for 10 min by occlusion of the aortic inflow, (iii) reperfused retrogradely
39
40 for 10 min following 10 min of global ischaemia, or (iv) perfused retrogradely with 40 nM
41
42 calyculin A for 5 min. The hearts were then frozen between tongs cooled in liquid N₂ and
43
44 powdered by grinding in a pestle and mortar cooled in liquid N₂.
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

2.7. Immunofluorescence staining

1
2
3
4
5 Myocytes were cultured on glass coverslips [precoated with laminin (20 µg/ml in
6
7 PBS) and 1% (w/v) gelatin] placed in 35 mm Primaria culture dishes at a density of 1.5×10^6
8
9 cells/dish for 18 h in the presence of 15% (v/v) foetal calf serum. Myocytes were infected
10
11 with Adv.FLAG-NDR2 (as described under '*Infection with adenoviral vectors*' above) for 16
12
13 h in serum-free medium. The medium was discarded and the cells were washed with ice-cold
14
15 PBS (3 × 0.5 ml). Cells were fixed in 4% (w/v) formaldehyde (10 min), permeabilised with
16
17 0.3% (v/v) Triton X-100 in PBS, and non-specific antibody binding was blocked with 10%
18
19 (w/v) horse serum in PBS containing 0.3% (v/v) Triton X-100. FLAG-NDR2 was detected
20
21 by incubation of cells with mouse anti-FLAG antibody (1:25 dilution, 2 h, 37°C), a
22
23 biotinylated goat anti-mouse IgG secondary antibody (1:200 dilution, 30 min, room
24
25 temperature) and streptavidin-Texas Red (1:200 dilution, 30 min, room temperature).
26
27 Sarcomeric structures were identified by FITC-phalloidin (1:100, 1 h, room temperature).
28
29 Nuclei were stained with Hoechst 33258 (50 µg/ml, 10 min, room temperature). Cells were
30
31 washed with PBS (3 × 0.5 ml) between each incubation. Slides were mounted and examined
32
33 using a Zeiss Axioskop microscope with a 100× oil immersion objective and photographed
34
35 using a digital camera.
36
37
38
39
40
41
42
43
44
45
46

2.8. Quantitative PCR (qPCR)

47
48
49
50
51 RNA was extracted and cDNA synthesised using reverse transcription as previously
52
53 described [31]. qPCR was performed using a Real-Time PCR System model 7500 (Applied
54
55 Biosystems). Amplifications were carried out in optical 96-well reaction-plates (Applied
56
57 Biosystems) with each well containing 12.5 µl of SYBR Green Jump Start Taq Readymix
58
59

1 (Sigma Aldrich Chemical Co.), 5 μ l of oligonucleotide primers (5 pmol each of forward and
2 reverse primers) and 7.5 μ l of cDNA template (diluted 15-fold in water). Details of the
3 primers used are given in Table 1. qPCR analysis of Gapdh was performed as a control and
4 the relative quantification protocol was used. PCR conditions for all primer pairs were 50°C
5 for 2 min, 95°C for 10 min (Jump-Start Taq polymerase activation step), followed by 40
6 cycles of 95°C for 15 s and 59°C for 60 s. Following qPCR, dissociation curve analysis was
7 routinely performed to check for aberrant amplification products (e.g. primer-dimers).
8
9

10 11 12 13 14 15 16 17 *2.9. Calculation of results and statistical methods* 18 19 20

21 Within a single experiment (i.e. a single preparation of cardiac myocytes), results are
22 expressed relative to the maximum signal for the quantified immunoblots or FLAG-NDR2
23 activities. Statistical significance was tested using two-tailed Student's *t* test for paired
24 samples or one-way ANOVA with Tukey's multiple comparison test as appropriate. A
25 statistically significant difference required that $P < 0.05$.
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

3. Results

3.1. Molecular cloning of rat NDR2 cDNA

In an Affymetrix rat 230 2.0 array screen of primary cultures of neonatal rat cardiac myocytes exposed to ET-1 for 4 h, two probe-sets (1372955_at and 1383299_at) identified an expressed sequence tag (EST) that was significantly upregulated to 2.28-fold (average of both probe-sets) of the zero-time control [18]. A BLAST search revealed that this rat EST corresponded to the mouse NDR2 nucleotide sequence. Rat NDR2 cDNA was cloned as described in 'Supplementary Material' and the sequence obtained (bases 1 - 2406) deposited in Genbank (accession no. EF444939.1). The translational open reading frame (bases 151 - 1542, 464 amino acids) was aligned against human, mouse and rat NDR1, and human and mouse NDR2 using the ClustalW program (www.ebi.ac.uk/clustalw) (Supplementary Material, Fig. 1). The primary structure of rat NDR2 (calculated molecular mass: 53.6 kDa) is identical to mouse NDR2 except for replacement of mouse Asn-15 and Val-216 with Ser-15 and Ile-216, respectively, in rat NDR2.

Using qPCR, we verified that NDR2 mRNA was significantly upregulated (3- to 4-fold of zero-time control) by ET-1 at 1.5 h and remained upregulated at this level until at least 8 h (Fig. 1A). In contrast, NDR1 mRNA was not significantly altered over the same time period (Fig. 1B). However, using the antibody which recognises both NDR1 and NDR2, we were unable to detect any change in the abundances of NDR proteins by ET-1 over the same time period (results not shown). The primary structures of NDR1 and NDR2 show a high degree of identity (Supplementary Material, Fig. 1) and the proteins are very similar in molecular mass (NDR1 is 54.0 kDa), so that it is possible that any newly-synthesised NDR2 would not necessarily be readily detectable against the pre-existing NDR1/NDR2 background.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

3.2. Phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 by calyculin A

Calyculin A is an inhibitor of PPP1 and PPP2 (formerly known as PP2A), inhibiting each approximately equipotently (Table 2). Calyculin A (200 nM) induced a rapid phosphorylation (detectable by 1 min) of NDR1(Thr-444)/NDR2(Thr-442) in cardiac myocytes, reaching a maximum at 10 – 20 min and remaining stable for at least 90 min (Fig. 2A). There was no change in total NDR1/NDR2 abundance (Fig. 2A). Because of their similarities in molecular mass, NDR1 and NDR2 usually co-migrate though, if samples are electrophoresed for longer, occasionally two closely-migrating bands are detectable with the antibody to NDR1(*P*Thr-444)/NDR2(*P*Thr-442). Using this anti-phospho-NDR antibody, the EC₅₀ for the calyculin A-mediated phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) was computed to be approximately 10 nM (Fig. 2B). The activation of NDR1 or NDR2 requires dual phosphorylation of NDR1(Ser-281/Thr-444) or NDR2(Ser-282/Thr-442), the phosphorylation of NDR1(Ser-281)/NDR2(Ser-282) representing autophosphorylations following the NDR1(Thr-444)/NDR2(Thr-442) phosphorylations [3,13,14]. However, the available antibody [14] raised against NDR1(*P*Ser-281)/NDR2(*P*Ser-282) is of relatively low affinity/efficacy and we could not examine the phosphorylation of these sites. We did investigate whether, following adenovirally-mediated transfer of FLAG-NDR2, the activity of FLAG-NDR2 as measured by immunoprecipitation kinase assays was commensurate with phosphorylation of NDR1(Thr-444)/NDR2(Thr-442). We adjusted the adenoviral infections so that we achieved essentially equal expression of FLAG-NDR2 and endogenous NDR1/NDR2 (Fig. 2C). Immunoprecipitation kinase assays using the anti-FLAG antibody showed that, as for the phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) (Fig. 2B), the activity of FLAG-NDR2 was also stimulated by calyculin A with an EC₅₀ of approximately 10 nM (Fig. 2D). We noticed that calyculin A also appeared to be positively chronotropic in these myocyte cultures and thus considered the possibility that contractile activity might be responsible for NDR phosphorylation. However, contractile arrest by blockade of the L-type-

1 and T-type Ca²⁺ channels with 10 μM nifedipine and 1.8 μM mibefradil did not reduce the
2 effects of calyculin A (results not shown).
3
4
5

6 *3.3. Phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) by hyperosmotic shock and* 7 8 *oxidative stress* 9

10
11
12 Exposure of myocytes to hyperosmotic shock (0.5 M sorbitol) resulted in a rapid
13 (maximal in 10 min or less) phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) (Fig. 3A)
14 though, unlike phosphorylation elicited by 200 nM calyculin A (Fig. 2A), phosphorylation
15 had declined by 90 min. Oxidative stress (0.5 mM H₂O₂) caused a somewhat slower
16 phosphorylation (maximal at about 20 min) and this again declined by 90 min (Fig. 3B). In
17 neither condition was there any change in the abundance of total NDR1/NDR2 (Fig. 3A-B).
18 The phosphorylation elicited by oxidative stress was detectable at 0.1 mM H₂O₂ and showed a
19 distinct peak occurring at about 0.5 mM (Fig. 3C). At higher concentrations (1 – 10 mM
20 H₂O₂), phosphorylation declined. There were no losses of myocytes from the dishes at up to
21 3 mM H₂O₂, as shown by the immunoblots against total NDR1/NDR2 (Fig. 3C), though it is
22 possible that some losses occurred at 10 mM H₂O₂.
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37

38 *3.4. Relative potencies of phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation* 39 *of FLAG-NDR2 by calyculin A, hyperosmotic shock and H₂O₂, and identification of* 40 *additional modulators* 41 42 43 44 45

46 We examined the ability of a wide range of known modulators of myocardial
47 processes to mediate phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and/or activation
48 of FLAG-NDR2 (see Supplementary Material, Table 2 for details of agonists/interventions
49 without effect). We also set any modulators identified as being effective in a hierarchy in
50 comparison with calyculin A. Chelerythrine is allegedly a protein kinase C (PKC) inhibitor
51 [32], though this has been questioned at least in the case of the PKCα isoform [33]. In
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 cardiac myocytes, chelerythrine causes conventional and novel PKC-independent pyknosis,
2 shrinkage and death that may result from the induction of oxidative stress [34]. In
3
4 comparison with 200 nM calyculin A (NDR1/NDR2 control not subtracted), chelerythrine is
5
6 about 45 to 50% as effective in inducing phosphorylation of NDR1(Thr-444)/NDR2(Thr-442)
7
8 (Fig. 4A), but is only about 20% (control FLAG-NDR2 activity not subtracted) as effective in
9
10 inducing activation of FLAG-NDR2 (Fig. 4B). Likewise, 0.5 mM H₂O₂ is about 40 to 45% as
11
12 effective as 200 nM calyculin A in inducing phosphorylation of NDR1(Thr-444)/NDR2(Thr-
13
14 442) but is only about 5 to 10% as effective in inducing activation of FLAG-NDR2 (Fig. 4A-
15
16 B). The PPP2 inhibitor okadaic acid (OKA), which also inhibits PPP1 though less effectively
17
18 (Table 2), is known to induce phosphorylation and activation of NDR1/NDR2 [3,12]. In our
19
20 hands, after a 60 min incubation, 1 μM OKA is almost as effective as incubation with 200
21
22 nM calyculin A for 20 or 60 min in inducing phosphorylation of NDR1(Thr-444)/NDR2(Thr-
23
24 442) (Fig. 4A). However, following a 20 min incubation, 1 μM OKA does not elicit any
25
26 statistically significant phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) compared with a
27
28 control incubation (Fig. 4A), though there is a statistically-insignificant increase to about 30%
29
30 of the level observed with 200 nM calyculin A for 20 or 60 min. For activation of FLAG-
31
32 NDR2, OKA (1 μM, 20 min) was <10% as effective as calyculin A (200 nM, 20 min) (Fig.
33
34 4C). However, after a 60 min incubation with OKA, this had increased to 45 to 50% (Fig.
35
36 4C). The general conclusions are (i) calyculin A is the most powerful stimulator of
37
38 NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 that we have identified, (ii)
39
40 generally, activation of FLAG-NDR2 lags behind phosphorylation of NDR1(Thr-
41
42 444)/NDR2(Thr-442). Because it could be argued that this difference was related to the
43
44 different species of NDRs examined (endogenous NDR1/NDR2 or FLAG-NDR2), we
45
46 examined the phosphorylation of endogenous NDR1/NDR2 and FLAG-NDR2 in FLAG-
47
48 NDR2-transduced cardiac myocytes (Fig. 4D). This was feasible because of the lower
49
50 mobility of FLAG-NDR2. No differences between the phosphorylation of endogenous
51
52 NDR1(Thr-444)/NDR2(Thr-442) and FLAG-NDR2(Thr-442) could be identified for any
53
54 agonist. We also calculated the approximate ratio of expression of FLAG-NDR2(*P*Thr-442)
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

to endogenous NDR1(*P*Thr-444)/NDR2(*P*Thr-442) using the anti-NDR1(*P*Thr-444)/NDR2(*P*Thr-442) antibody. If it can be assumed that the most significant factor influencing this ratio is the levels of expression, the value should approximate to the FLAG-NDR2/endogenous NDR1/NDR2 ratio (Fig. 2C). The FLAG-NDR2(*P*Thr-442)/endogenous NDR1(*P*Thr-444)/NDR2(*P*Thr-442) ratio is approximately 1.5, i.e. similar to that observed in Fig. 2C.

Given that calyculin A is an equipotent PPP1/PPP2 inhibitor whereas OKA inhibits PPP2 more potently (Table 2), the spectrum of NDR1/NDR2 phosphorylation (Fig. 4A,D) and FLAG-NDR2 activation (Fig. 4C) suggests that the effects of calyculin A could be exerted primarily through PPP1. We therefore investigated the effects of tautomycin (200 nM for up to 4 h) which is primarily a PPP1 inhibitor (Table 2) and fostriecin (200 nM for up to 4 h) which is primarily a PPP2 inhibitor (Table 2) on activity of FLAG-NDR2. Neither inhibitor had any detectable effect (results not shown). Furthermore, the potent PPP1/PPP2 inhibitor (Table 2), microcystin LR (2 μ M), did not activate FLAG-NDR2, nor did it induce phosphorylation of endogenous NDR1(Thr-444)/NDR2(Thr-442) or FLAG-NDR2(Thr-442). The effects of PPP inhibitors on the NDRs are therefore limited largely to calyculin A, with lesser effects of OKA.

3.5. *Inhibitors of calyculin A-mediated activation of FLAG-NDR2*

We investigated the ability of a number of known protein kinase inhibitors to reduce the activation of FLAG-NDR2 resulting from exposure of cardiac myocytes to 50 nM calyculin A. Of the inhibitors examined, the only inhibitor with any effect was staurosporine. Staurosporine was originally identified as a PKC inhibitor [35] but is now recognised to be a non-selective protein kinase inhibitor [36] (see also Reference [33] for 7-hydroxystaurosporine). Staurosporine exhibited an IC_{50} of approximately 40 nM for the inhibition of the activation of FLAG-NDR2 by 50 nM calyculin A (Fig. 5A). This inhibition

1 was not attributable to 'carry-over' of staurosporine from the cell incubations into the assay
2 because addition of staurosporine up to 1 μ M for the last 5 min of a 20 min exposure of
3 myocytes to 50 nM calyculin A [i.e. after FLAG-NDR2 would have been essentially
4 completely activated by calyculin A (Fig. 2A) but prior to extraction and
5 immunoprecipitation] did not result in any significant reduction in FLAG-NDR2 activity. It
6 is possible that staurosporine may not inhibit phosphorylation of NDR1(Thr-444)/NDR2(Thr-
7 442) but could selectively inhibit the autophosphorylation of NDR1(Ser-281)/NDR2(Ser-
8 282). We therefore also examined its effects on the calyculin A-stimulated phosphorylation
9 of NDR1(Thr-444)/NDR2(Thr-442) and FLAG-NDR2(Thr-442) (Fig. 5B). Staurosporine
10 also inhibited the calyculin A-stimulated phosphorylation of endogenous NDR1(Thr-
11 444)/NDR2(Thr-442) and of exogenous FLAG-NDR2(Thr-442) (Fig. 5B), and this in itself
12 should prevent phosphorylation of FLAG-NDR2(Ser-282) and its activation (Fig. 5A). We
13 do not know whether staurosporine also inhibits the autophosphorylation of NDR1(*P*Thr-
14 444)/NDR2(*P*Thr-442) at the T loop residues.

15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31 The following were ineffective in inhibiting activation of FLAG-NDR2 by calyculin
32 A: 100 μ M genistein (a generalised Tyr-protein kinase inhibitor [37]), 10 μ M GF109203X
33 (generalised PKC inhibitor [38] which also inhibits a number of other protein kinases [33]),
34 20 μ M HA1077 (a PKN inhibitor [39] which inhibits a number of protein kinases including
35 protein kinase A and Rho kinase [33]), 10 μ M SB203580 [inhibitor of the α and β isoforms
36 of p38-mitogen-activated protein kinase (p38-MAPK) [40]] or 10 μ M Y27632 (Rho kinase
37 inhibitor [41] which also inhibits PKN [33]). Equally, no inhibitor activated FLAG-NDR2 in
38 the absence of calyculin A (results not shown). The phosphoinositide 3-kinase (PI3K)
39 inhibitor, LY294002 [42] (50 μ M), did not inhibit NDR1(Thr-444)/NDR2(Thr-442)
40 phosphorylation or activation of FLAG-NDR2 (results not shown). [We ensured that the
41 LY294002 preparation used was active by demonstrating that it prevented stimulation of
42 protein kinase B/Akt (PKB/Akt) phosphorylation by 300 nM insulin.]
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

3.6. Activity of FLAG-NDR2-PIFtide

1
2
3
4 Mutation of the two phosphorylation sites in the catalytic and hydrophobic domains of
5 NDR1 (i.e. Ser-281 and Thr-444) to acidic residues is relatively-ineffective in producing a
6 kinase with increased constitutive activity [3]. A constitutively-activated species can be
7 produced by splicing NDR2(1-432) to PKN2(969-983) to produce NDR2-PIFtide, as has been
8 previously described for human NDR2 [3]. We examined the activities of FLAG-NDR2 (rat)
9 and FLAG-NDR2-PIFtide (rat-human chimera) in the absence or presence of calyculin A and
10 approximately matching the abundances of FLAG-NDR2 and FLAG-NDR2-PIFtide (Fig. 6).
11 After subtracting the activity seen following infection with the adenoviral blank (which was
12 not accurately matched for multiplicity of infection with Adv.FLAG-NDR2 and Adv.FLAG-
13 NDR2-PIFtide) and correcting for differences in relative abundance, the constitutive activity
14 of FLAG-NDR2-PIFtide was found to be approximately 10-fold greater than that of FLAG-
15 NDR2 (Fig. 6). Not unexpectedly, FLAG-NDR2-PIFtide (which lacks the Thr-442
16 phosphorylation site) could not be activated by calyculin A (Fig. 6).
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31

3.7. Intracellular localisation of NDR2 in cardiac myocytes

32
33
34 Using the antibodies available, we were unsuccessful in staining for endogenous
35 dephospho-NDR (as were Hergovich *et al.* [16] in U2-OS cells), nor were we successful in
36 staining for phospho-NDRs. We therefore examined the intracellular localisation of NDR2
37 by infecting myocytes with Adv.FLAG-NDR2 and staining for the FLAG epitope. FLAG-
38 NDR2 localised to the cytoplasm in cardiac myocytes and was clearly excluded from the
39 nucleus (Fig. 7). The pattern of staining was somewhat uneven in the cytoplasm and there
40 was a suggestion of some partition of NDR2 to the plasma membrane. There was no
41 evidence of any change in localisation following exposure to 30 nM calyculin A for 20 min
42 (results not shown).
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

3.8. Phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) in intact heart by calyculin A or by ischaemia and reperfusion

Calyculin A proved to be the most powerful stimulator of NDR1(Thr-444)/NDR2(Thr-442) phosphorylation and activator of FLAG-NDR2 in neonatal rat cardiac myocytes that we identified here (see Fig. 4A-D). Some of the interventions resulting in phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 in myocytes are established cytotoxic stresses in neonatal cardiac myocytes (chelerythrine [34], H₂O₂ [43] and hyperosmotic shock [44]). However, it is important to show that these findings are not confined to neonatal cells. We therefore investigated the effects of calyculin A and of pathologically-relevant cardiac stresses, namely ischaemia and ischaemia-reperfusion in intact adult rat hearts. As expected, calyculin A (40 nM, 5 min) induced a highly-significant ($P < 0.0001$) increase in the phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) in perfused hearts (Fig. 8). Although there was some suggestion that global ischaemia (10 min) increases phosphorylation of NDR1(Thr-444)/NDR2(Thr-442), this was not significantly different from control normoxic perfusions. In contrast, global ischaemia (10 min) followed by reperfusion (10 min) resulted in significant ($P < 0.0001$) phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) which, after subtraction of the control, was equivalent to about 30% of the level induced by 40 nM calyculin A for 5 min (Fig. 8).

4. Discussion

4.1. General comments

The NDRs are highly-conserved protein Ser-/Thr-kinases that are members of the AGC kinase superfamily [1,2]. In terms of their activation, the current scheme is that NDR1(Thr-444) and NDR2(Thr-442) (the hydrophobic domain residues) are first phosphorylated by an upstream kinase and then NDR1(*P*Thr-444) and NDR2(*P*Thr-442) each autophosphorylate their own T loop residues in their catalytic domains to produce the active species NDR1(*P*Ser-281/*P*Thr-444) and NDR2(*P*Ser-282/*P*Thr-442) [1,2]. The fact that activation of FLAG-NDR2 tends to lag behind phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) (for example, seen clearly for OKA in Fig. 4A, C) suggests that this ordered phosphorylation occurs in cardiac myocytes. The agonists that lead to activation of NDRs are poorly-characterised and their biological functions in higher organisms are poorly-understood. Given that NDR1/NDR2 are highly-conserved through evolution, it seems likely that they possess important functions. NDR2 may be involved in organisation of the actin cytoskeleton [9] and, recently, a role for NDR1/NDR2 in centrosome duplication has been identified [2,10]. Although populations of myocytes range from the mononucleate to the multinucleate and thus must possess some capacity for karyokinesis, they are generally considered to be terminally-differentiated and incapable of cytokinesis. It therefore seems unlikely that centrosome duplication is of major importance in their case.

4.2. Expression of the *NDR1* and *NDR2* genes

At the level of mRNA, NDR2 transcripts are rapidly but transiently induced in amygdalae of mice following emotional stress [9] and, here, we show that NDR2 mRNA is also rapidly induced by ET-1 in neonatal rat cardiac myocytes (Fig. 1A). In contrast, NDR1 mRNA is not induced by ET-1 (Fig. 1B). Presumably, their promoter regions are sufficiently

1 different to allow differential transcription or else transcript stability may be involved. Using
2 microarrays (Affymetrix rat 230 2.0 array at 30 min or the more-restricted Affymetrix rat
3 U34A microarrays at 2 or 4 h) in conjunction with semi-quantitative PCR and/or qPCR, we
4 have shown that, for ET-1, the activation of the ERK1/2 cascade is required for the increased
5 expression of the majority of transcripts upregulated at 30 min, 2 h or 4 h [18,24]. We have
6 not yet studied in detail whether this is the case for NDR2 mRNA [the U34A microarrays did
7 not have a probe set for rat NDR2 [24] and we only studied the 30 min time point in our more
8 recent study [18] when NDR2 transcripts are not upregulated (Fig. 1A)].
9

10 11 12 13 14 15 16 17 18 19 *4.3. Involvement of PPPs in NDR phosphorylation and activation*

20
21
22
23 The PPP superfamily, of which there are seven sub-families, is encoded by 20 genes
24 in *Homo sapiens* and these encode about 28 catalytic subunits [45]. There is also a
25 multiplicity of regulatory subunits (particularly for PPP1) which are involved in targeting (to
26 control subcellular localisation and substrate specificity) and regulation (e.g. inhibitory
27 subunits). Previously, OKA has been identified as an agent that stimulates phosphorylation
28 and activation of NDR1/NDR2 [3,12]. This is presumably achieved by inhibiting a PPP,
29 allowing unknown NDR1/NDR2 kinases to phosphorylate NDRs. Here, we show that, at
30 maximally-effective concentrations, calyculin A is more effective than OKA in inducing
31 NDR1(Thr-444)/NDR2(Thr-442) phosphorylation and activation of FLAG-NDR2 in neonatal
32 rat cardiac myocytes after 20 min (Fig. 4A, C). Although calyculin A or OKA treatment
33 result in equivalent NDR1(Thr-444)/NDR2(Thr-442) phosphorylation after 60 min, activation
34 of FLAG-NDR2 with OKA is still less than with calyculin A (Fig. 4A, C). A similar
35 hysteresis between phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of
36 FLAG-NDR2 is seen in the individual comparisons of all interventions (Fig. 4). It probably
37 reflects the ordered nature of the phosphorylation of first NDR1(Thr-444)/NDR2(Thr-442)
38 followed by NDR1(Ser281)/NDR2(Ser-282). In previous work in which COS cells were
39 transfected with tagged NDRs, OKA (1 μ M, 60 min) stimulated NDR activity by 12- to 25-

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

fold [3,12]. This compares with 21-fold in our analogous experiments (Fig. 4C), but we find that calyculin A (200 nM, 60 min) stimulates activity by about 45-fold (Fig. 4C). We do not know whether these differences result from trivial causes (e.g. differences in permeability) or whether there are mechanistic differences. Importantly, calyculin A stimulates phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) in adult perfused heart (Fig. 8), confirming that the findings in neonatal myocyte are not confined this particular stage of maturation.

16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Though calyculin A and OKA may show differing selectivities for PPP1 and PPP2 [OKA has a 50-fold greater IC_{50} for PPP1 than PPP2 (Table 2)], they are both relatively potent inhibitors of both PPPs at least *in vitro* (Table 2). We had hoped possibly to discriminate between PPP1 and PPP2 using the more selective inhibitors fostriecin and tautomycetin (Table 2), and we also studied the effects of microcystin LR. However, we were never able to activate FLAG-NDR2 with these, nor did microcystin LR cause phosphorylation of endogenous NDR1(Thr-444)/NDR2(Thr-442) or FLAG-NDR2(Thr-442). We do not understand the reasons for this. They could be trivial (e.g. permeability), but all ineffective inhibitors have been used in intact cell preparations previously. Equally, there may be PPP1/PPP2 holoenzyme species which are insensitive to fostriecin, tautomycetin or microcystin LR, or there may be PPP species other than PPP1 and PPP2 which are sensitive to calyculin A and OKA but which are not sensitive to fostriecin, tautomycetin or microcystin LR. Furthermore, it may be incorrect to assume that the actions of calyculin A or OKA are exerted at the level of the PPP(s) directly responsible for dephosphorylating NDRs. The point(s) of action could lie further upstream. The current predominating view that PPP2 is responsible for the dephosphorylation of NDR1(*PSer*-281/*PThr*-444) and NDR2(*PSer*-282/*PThr*-442) is based on experiments in intact cells with OKA [3,12], and on *in vitro* experiments involving dephosphorylation of NDR1(*PSer*-281/*PThr*-444) with recombinant PPP2 [12]. However, the concentration of OKA used in these experiments was 1 μ M or 300-times greater than the IC_{50} even for PPP1. Whilst there may be unassessed permeability

1 problems related to the access of OKA to the intracellular compartment, these experiments
2 are not unequivocal. Equally, *in vitro* experiments with recombinant PPP2 are not
3 unequivocal because of the possibility that any PPP might dephosphorylate the phospho-
4 NDRs if present in high enough excess. The PPP(s) responsible for dephosphorylating
5 NDR1(PSer-281/PThr-444) and NDR2(PSer-282/PThr-442) remain somewhat obscure.
6
7
8
9

10 11 12 4.4. Phosphorylation and activation of NDR1/NDR2 13 14 15

16 Here, we have shown that NDR1/NDR2 are phosphorylated and activated by a variety
17 of stimuli in cardiac myocytes and intact heart. Though we have not identified the kinases
18 responsible, these experiments show that the endogenous upstream signalling is intact. With
19 respect to the established kinases, PDK1 is often involved in phosphorylation of a T loop Ser-
20 /Thr-residue in AGC kinases, i.e. a Ser-/Thr-residue in the catalytic domain [46,47].
21
22

23 Activation of PDK1, which is probably potentially active in the basal state by virtue of its
24 high affinity interactions with 3-phosphoinositides [48], is mediated by PI3K-dependent
25 phosphorylation of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] to PtdIns(3,4,5)P₃
26 and lies downstream from the insulin or insulin-like growth factor 1 (IGF1) receptor protein
27 tyrosine kinases (RPTKs) as well as other RPTKs [47,49]. These T loop phosphorylations
28 are either regulatory (i.e. respond to external stimuli to change phosphorylation state and
29 activity of the kinase involved) or are essentially facilitative (i.e. are constitutive rather than
30 regulatory, but are necessary for activity). Evidence has been presented that neither PDK1
31 nor insulin or IGF1 is involved in the regulation of NDR1 [12] though, equally, contradictory
32 evidence has been presented that IGF1-activated PDK1 is an NDR2(Thr-442) kinase and this
33 phosphorylation precedes the autophosphorylation of the T loop residue [15]. We did not
34 find any evidence that insulin (which does cause PI3K/PDK1-dependent phosphorylation of
35 PKB/Akt in cardiac myocytes [50]) stimulated phosphorylation of NDR1(Thr-
36 444)/NDR2(Thr-442) (Supplementary Material, Table 2A) nor did LY294002 inhibit the
37 phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) or activation of FLAG-NDR2 by
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 calyculin A. Our data thus do not support a role for PDK1. A second mode of activation
2 proposed by Suzuki *et al.* [15] involves a S100/Ca²⁺-dependent phosphorylation of
3 NDR2(Thr-75), then phosphorylation of NDR2(Thr-442) in the NDR2(PThr-75) species by
4 an unknown kinase, and finally autophosphorylation of NDR2(Ser-281) in the NDR2(PThr-
5 75/PThr-442) species leading to activation [14,15]. The necessary increase in Ca²⁺
6 concentrations is mediated through phospholipase C γ (PLC γ) and hydrolysis of PtdIns(4,5)P₂
7 to the Ca²⁺-mobilising ‘second messenger’, inositol 1,4,5-*tris*phosphate [Ins(1,4,5)P₃] [15].
8 However, in cardiac myocytes, platelet-derived growth factor stimulates PtdIns(4,5)P₂
9 hydrolysis and Tyr-phosphorylation of PLC γ 1 [50], but does not stimulate NDR1(Thr-
10 444)/NDR2(Thr-442) phosphorylation (Supplementary Material, Table 2A). Furthermore,
11 ET-1 stimulates PLC β -mediated hydrolysis of PtdIns(4,5)P₂ in cardiac myocytes [51] but
12 does not stimulate phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) or activation of
13 FLAG-NDR2 (Supplementary Material, Table 2A and 2B).
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30

31 One upstream kinase that may be involved in the phosphorylation of NDR1(Thr-
32 444)/NDR2(Thr-442) is mammalian STE20-like kinase 3 (MST3) [13], also known as
33 STK24, a member of the germinal centre kinase family GCK-III subclass [52,53]. The
34 regulation of these kinases in terms of the agonists that lead to their activation is not well-
35 understood and indeed Pombo *et al.* [53] state that ‘To date, no stimuli have been shown to
36 activate MST3’. In fact, like the NDRs, MST3 is activated by OKA [13] and calyculin A
37 [54]. Overexpression of MST3 in HEK293 cell causes fragmentation of DNA indicative of
38 apoptosis/cell death [55]. If the NDRs are involved, this would suggest that their activation
39 by MST3 should be pro-apoptotic. It will be of interest to examine whether, in addition to
40 calyculin A and OKA, pro-apoptotic stimuli such as chelerythrine, hyperosmotic shock and
41 H₂O₂ activate MST3. One characteristic of the upstream NDR1(Thr-444)/NDR2(Thr-442)
42 kinase that we did identify is that it is inhibited by staurosporine (Fig. 5A-B). Staurosporine
43 and its derivatives inhibit a broad spectrum of Ser-/Thr-protein kinases (including PDK1),
44 though a number of kinases are relatively insensitive [33,36]. A prerequisite of the
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

NDR1(Thr-444)/NDR2(Thr-442) kinase is that its activity or activation should be inhibited by staurosporine.

4.5. *Pathophysiological relevance of interventions which stimulate phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2*

The interventions which stimulate phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 most clearly are calyculin A and OKA (Fig. 2A-B, D, Fig. 8) and OKA (Fig. 4A, C). Other than possibly contributing to the understanding of diarrhetic shellfish poisoning for which OKA is responsible, these findings have very little pathophysiological significance. The phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 are also stimulated by a relatively-limited subset of cell stress stimuli, notably hyperosmotic shock (Fig. 3A, Fig. 4A-B) and oxidative stress (Fig. 3B-C, Fig. 4A-B) in neonatal cardiac myocytes. Perhaps most notably, global ischaemia/reperfusion, a pathologically-important pro-apoptotic stress in the heart [56], stimulates phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) in isolated adult rat hearts (Fig. 8). Although there is a suggestion that ischaemia alone also resulted in phosphorylation of NDR1(Thr-444)/NDR2(Thr-442), this result did not achieve statistical significance (Fig. 8). In isolated hearts, the stress-activated protein kinases of the MAPK family are activated (by phosphorylation) by ischaemia and ischaemia/reperfusion [57]. Thus, ischaemia activates p38-MAPKs and this is increased on reperfusion [56,57]. Ischaemia alone does not activate the c-Jun N-terminal kinases (JNKs), but they are activated on reperfusion [57]. However, p38-MAPKs and JNKs are activated by a wider range of cellular stresses (e.g. anisomycin) in cardiac myocytes than those that lead to phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) (Supplementary Material, Table 2A) or activation of FLAG-NDR2 (Supplementary Material, Table 2B), and they are also activated by some G protein receptor agonists, e.g. ET-1 and phenylephrine [58-60]. As with phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) and activation of FLAG-NDR2 (Fig. 3B-C, Fig. 4A-B), JNKs and p38-MAPKs are activated by

1 oxidative stress in cardiac myocytes and the perfused heart [30,61], and our findings indicate
2 that the increases in oxidative stress that the heart experiences during ischaemia and, more
3 particularly, on reperfusion [62] contribute to their activation [30]. A similar mechanism
4 could apply to the NDRs.
5
6
7
8
9

10 4.6. Subcellular localisation of NDR2 11 12 13

14 The subcellular localisation of the NDRs as assessed by either immunofluorescence or
15 subcellular fractionation is controversial. NDR1 was first thought to be localised to the
16 nucleus [5]. Tagged NDR1 overexpressed in HeLa cells also appears to localise primarily to
17 the nucleus with evidence of an additional cytoplasmic localisation at higher levels of
18 expression [4]. Somewhat surprisingly in view of the similarity in their primary sequences
19 (see Supplementary Material, Fig. 1), endogenous or overexpressed tagged NDR2 appears to
20 localise primarily to the cytoplasm (sometimes in a punctate manner) with evidence of
21 nuclear localisation or perinuclear concentration when expression at higher levels [3,4].
22 However, Hergovich *et al.* [16] (see also Reference [10]) have now demonstrated that both
23 endogenous NDR1 and overexpressed NDR1 are primarily cytoplasmic and suggest that
24 some previous studies demonstrating a nuclear localisation resulted from misinterpretation of
25 immunofluorescence data. In our hands, FLAG-NDR2 expressed ectopically to about the
26 same level as total endogenous NDRs localises to the cytoplasm of cardiac myocytes, though
27 there is some evidence of increased concentration at the plasma membrane (Fig. 7). This
28 might perhaps be in keeping with the need for NDR2, on association with MOB proteins, to
29 translocate to the plasma membrane for activation by phosphorylation [11,16]. We did not
30 assess the localisation of NDR1.
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

4.7. NDR2-PIFtide

1
2
3
4 When protein kinases are activated by Ser-/Thr- phosphorylation, the normal tactic to
5 increase the level of constitutive activity is to mutate the Ser-/Thr- residues to Asp- or Glu-
6 However, mutation of NDR1(Ser-281) does not affect activity and mutation of NDR1(Thr-
7 444) caused only a 1.5- to 2-fold increase in activity [12]. In PKB/Akt, phosphorylation of
8 the homologous hydrophobic domain Ser- (Ser-472, Ser-473 or Ser-474) depending on the
9 isoform (α , β or γ) results in full activation of PKB/Akt phosphorylated on the PDK1 site in
10 the T loop (Thr-308 or Thr-309) [49]. Crystallographic studies show that phosphorylation of
11 PKB β /Akt2(Ser-474) results in a disorder-to-order transition of the α C helix and interaction
12 with PKB/Akt(*P*Thr-309) [63]. This ordered structure can be mimicked by the hydrophobic
13 motif C-terminal region of PKN2 which contains the so-called PIFtide sequence which is
14 highly ordered [64]. In NDR2, replacement of residues 433-464 with the PKN2 PIF domain
15 (human PKN2 residues 969-983) produces an NDR2 species which was estimated as
16 possessing a 20-fold increase in activity over the wild type NDR2 and in which the T loop
17 phosphorylation site is phosphorylated [3]. The specific activity of tagged NDR2-PIFtide is
18 about 2-fold greater than that of the tagged NDR2(wild type) from COS cells treated with 1
19 μ M OKA for 60 min, and its activity might be slightly further increased by exposure of the
20 cells to OKA [3]. We estimate here that the activity of FLAG-NDR2-PIF-tide was about 10-
21 fold greater than FLAG-NDR2(wild-type) (Fig. 6), and this was still much less (about 15-
22 20%) than that of FLAG-NDR2(wild-type) that had been activated by 200 nM calyculin A for
23 20 min. Calyculin A did not further activate FLAG-NDR2-PIFtide (Fig. 6), presumably
24 because Thr-442 is absent. We were hoping that ectopic expression of the activated FLAG-
25 NDR2-PIFtide might cause some obvious changes in its subcellular localisation and/or
26 myocyte morphology. However, we were not able to detect any such changes and a more-
27 detailed examination of any prospective phenotypes is currently being undertaken.
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Acknowledgements

1
2
3
4 This work was supported by Fondation Leducq ‘Transatlantic Network of Excellence’
5
6 Grant 05-CVD2 to P.H.S., A.C. and J.D.M., and by British Heart Foundation Grants
7
8 RG/2001007 and PG/07/060/23276 to P.H.S. and A.C. S.P. was supported by Fellowships
9
10 from the Sigrid Jusélius Foundation, the Finnish Foundation for Cardiovascular Research, the
11
12 Instrumentarium Foundation, the Aarne and Aili Turunen Foundation, the Maud Kuistila
13
14 Foundation and the Paavo Nurmi Foundation. A.H was supported by Oncosuisse Grant OCS
15
16 1942-08-2006. Research at the Friedrich Miescher Institute is supported by the Novartis
17
18 Research Foundation.
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

References

- 1
2
3
4 [1] A. Hergovich, M.R. Stegert, D. Schmitz, B.A. Hemmings, *Nat. Rev. Mol. Cell Biol.* 7
5
6 (2006) 253.
7
8 [2] A. Hergovich, H. Cornils, B.A. Hemmings, *Biochim. Biophys. Acta* 1784 (2008) 3.
9
10 [3] M.R. Stegert, R. Tamaskovic, S.J. Bichsel, A. Hergovich, B.A. Hemmings, *J. Biol.*
11
12 *Chem.* 279 (2004) 23806.
13
14 [4] E. Devroe, H. Erdjument-Bromage, P. Tempst, P.A. Silver, *J. Biol. Chem.* 279 (2004)
15
16 24444.
17
18 [5] T. Millward, P. Cron, B.A. Hemmings, *Proc. Natl. Acad. Sci. U. S. A.* 92 (1995) 5022.
19
20 [6] T.A. Millward, C.W. Heizmann, B.W. Schäfer, B.A. Hemmings, *EMBO J.* 17 (1998)
21
22 5913.
23
24 [7] A.S. Mah, A.E. Elia, G. Devgan, J. Ptacek, M. Schutkowski, M. Snyder, M.B. Yaffe, R.J.
25
26 Deshaies, *BMC Biochem.* 6 (2005) 22.
27
28 [8] Y. Hao, A. Chun, K. Cheung, B. Rashidi, X. Yang, *J. Biol. Chem.* 283 (2008) 5496.
29
30 [9] O. Stork, A. Zhdanov, A. Kudersky, T. Yoshikawa, K. Obata, H.-C. Pape, *J. Biol. Chem.*
31
32 *279* (2004) 45773.
33
34 [10] A. Hergovich, S. Lamla, E.A. Nigg, B.A. Hemmings, *Mol. Cell* 25 (2007) 625.
35
36 [11] S.J. Bichsel, R. Tamaskovic, M.R. Stegert, B.A. Hemmings, *J. Biol. Chem.* 279 (2004)
37
38 35228.
39
40 [12] T.A. Millward, D. Hess, B.A. Hemmings, *J. Biol. Chem.* 274 (1999) 33847.
41
42 [13] M.R. Stegert, A. Hergovich, R. Tamaskovic, S.J. Bichsel, B.A. Hemmings, *Mol. Cell.*
43
44 *Biol.* 25 (2005) 11019.
45
46 [14] R. Tamaskovic, S.J. Bichsel, H. Rogniaux, M.R. Stegert, B.A. Hemmings, *J. Biol.*
47
48 *Chem.* 278 (2003) 6710.
49
50 [15] A. Suzuki, T. Ogura, H. Esumi, *J. Biol. Chem.* 281 (2006) 13915.
51
52 [16] A. Hergovich, S.J. Bichsel, B.A. Hemmings, *Mol. Cell. Biol.* 25 (2005) 8259.
53
54 [17] M.C. Hou, D.A. Guertin, D. McCollum, *Mol. Cell. Biol.* 24 (2004) 3262.
55
56
57
58
59
60
61
62
63
64
65

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [18] T.E. Cullingford, T. Markou, S.J. Fuller, A. Giraldo, S. Pikkarainen, G. Zoumpoulidou, A. Alsafi, C. Ekere, T.J. Kemp, J.L. Dennis, L. Game, P.H. Sugden, A. Clerk, *Genome Biol.* 9 (2008) R32[.
- [19] H.E. Shubeita, P.M. McDonough, A.N. Harris, K.U. Knowlton, C.C. Glembotski, J.H. Brown, K.R. Chien, *J. Biol. Chem.* 265 (1990) 20555.
- [20] H. Ito, Y. Hirata, M. Hiroe, M. Tsujino, S. Adachi, T. Takamoto, M. Nitta, K. Taniguchi, F. Marumo, *Circ. Res.* 69 (1991) 209.
- [21] P.H. Sugden, *J. Mol. Cell. Cardiol.* 35 (2003) 871.
- [22] M.A. Bogoyevitch, P.E. Glennon, P.H. Sugden, *FEBS Lett.* 317 (1993) 271.
- [23] M.A. Bogoyevitch, P.E. Glennon, M.B. Andersson, A. Clerk, A. Lazou, C.J. Marshall, P.J. Parker, P.H. Sugden, *J. Biol. Chem.* 269 (1994) 1110.
- [24] R.A. Kennedy, T.J. Kemp, P.H. Sugden, A. Clerk, *J. Mol. Cell. Cardiol.* 41 (2006) 236.
- [25] A. Clerk, T.E. Cullingford, S.J. Fuller, A. Giraldo, T. Markou, S. Pikkarainen, P.H. Sugden, *J. Cell. Physiol.* 212 (2007) 311.
- [26] K. Iwaki, V.P. Sukhatme, H.E. Shubeita, K.R. Chien, *J. Biol. Chem.* 265 (1990) 13809.
- [27] M.A. Bogoyevitch, A. Clerk, P.H. Sugden, *Biochem. J.* 309 (1995) 437.
- [28] M.M. Bradford, *Anal. Biochem.* 72 (1976) 248.
- [29] A. Clerk, M.A. Bogoyevitch, M.B. Andersson, P.H. Sugden, *J. Biol. Chem.* 269 (1994) 32848.
- [30] A. Clerk, S.J. Fuller, A. Michael, P.H. Sugden, *J. Biol. Chem.* 273 (1998) 7228.
- [31] T.J. Kemp, H.C. Causton, A. Clerk, *Biochem. Biophys. Res. Commun.* 307 (2003) 416.
- [32] J.M. Herbert, J.M. Augereau, J. Gleye, J.P. Maffrand, *Biochem. Biophys. Res. Commun.* 172 (1990) 993.
- [33] S.P. Davies, H. Reddy, M. Caivano, P. Cohen, *Biochem. J.* 351 (2000) 95.
- [34] S. Yamamoto, K. Seta, C. Morisco, S.F. Vatner, J. Sadoshima, *J. Mol. Cell. Cardiol.* 33 (2001) 1829.
- [35] T. Tamaoki, H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto, F. Tomita, *Biochem. Biophys. Res. Commun.* 135 (1986) 397.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [36] F. Meggio, A. Donella Deanna, M. Ruzzene, A.M. Brunati, L. Cesaro, B. Guerra, T. Meyer, H. Mett, D. Fabbro, P. Furet, G. Dobrowolska, L.A. Pinna, *Eur. J. Biochem.* 234 (1995) 317.
- [37] T. Akiyama, J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh, M. Shibuya, Y. Fukami, *J. Biol. Chem.* 262 (1987) 5592.
- [38] D. Toullec, P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle, L. Duhamel, D. Charon, J. Kirilovsky, *J. Biol. Chem.* 266 (1991) 15771.
- [39] N.E. Torbett, A. Casamassima, P.J. Parker, *J. Biol. Chem.* 278 (2003) 32344.
- [40] S. Kumar, P.C. McDonnell, R.J. Gum, A.T. Hand, J.C. Lee, P.R. Young, *Biochem. Biophys. Res. Commun.* 235 (1997) 533.
- [41] M. Uehata, S. Ishizaki, H. Satoh, T. Ono, T. Kawahara, T. Morishita, H. Tamakawa, K. Yamagami, J. Inui, M. Maekawa, S. Narumiya, *Nature* 389 (1997) 990.
- [42] C.J. Vlahos, W Matter,F,, K.Y. Hui, R.F. Brown, *J. Biol. Chem.* 269 (1994) 5241.
- [43] S.A. Cook, P.H. Sugden, A. Clerk, *Circ. Res.* 85 (1999) 940.
- [44] M. Galvez, M.P. Morales, J.M. Eltit, P. Ocaranza, L. Carrasco, X. Campos, M. Sapag-Hagar, G. Díaz-Araya, S. Lavandero, *Cell Tissue Res.* 304 (2001) 279.
- [45] M. Gallego, D.M. Virshup, *Curr. Opin. Cell Biol.* 17 (2005) 197.
- [46] A.C. Newton, *Biochem. J.* 370 (2003) 361.
- [47] A. Mora, D. Komander, D.M.F. van Aalten, D.R. Alessi, *Semin. Cell Dev. Biol.* 15 (2004) 161.
- [48] R.A. Currie, K.S. Walker, A. Gray, M. Deak, A. Casamayor, C.P. Downes, P. Cohen, D.R. Alessi, J. Lucocq, *Biochem. J.* 337 (1999) 575.
- [49] B. Vanhaesebroeck, D.R. Alessi, *Biochem. J.* 346 (2000) 561.
- [50] A. Clerk, I.-K.S. Aggeli, K. Stathopoulou, P.H. Sugden, *Cell. Signal.* 18 (2006) 225.
- [51] A. Clerk, P.H. Sugden, *J. Mol. Cell. Cardiol.* 29 (1997) 1593.
- [52] I. Dan, N.M. Watanabe, A. Kusumi, *Trends Cell Biol.* 11 (2001) 220.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [53] C.M. Pombo, T. Force, J. Kyriakis, E. Nogueira, M. Fidalgo, J. Zalvide, *Front. Biosci.* 12 (2007) 850.
- [54] K. Schinkmann, J. Blenis, *J. Biol. Chem.* 272 (1997) 28695.
- [55] C.-F.Y. Huang, Y.-M. Wu, C.-Y. Hsu, W.-S. Lee, M.-D. Lai, T.-J. Lu, C.-L. Huang, T.-H. Leu, H.-M. Shih, H.-I. Fang, D.R. Robinson, H.-J. Kung, C.-J. Yuan, *J. Biol. Chem.* 277 (2002) 34367.
- [56] X.L. Ma, S. Kumar, F. Gao, C.S. Loudon, B.L. Lopez, T.A. Christopher, C. Wang, J.C. Lee, G.Z. Feuerstein, T.L. Yue, *Circulation* 99 (1999) 1685.
- [57] M.A. Bogoyevitch, J. Gillespie-Brown, A.J. Ketterman, S.J. Fuller, R. Ben-Levy, A. Ashworth, C.J. Marshall, P.H. Sugden, *Circ. Res.* 79 (1996) 162.
- [58] M.A. Bogoyevitch, A.J. Ketterman, P.H. Sugden, *J. Biol. Chem.* 270 (1995) 29710.
- [59] A. Clerk, A. Michael, P.H. Sugden, *Biochem. J.* 333 (1998) 581.
- [60] A. Lazou, P.H. Sugden, A. Clerk, *Biochem. J.* 332 (1998) 459.
- [61] A. Clerk, A. Michael, P.H. Sugden, *J. Cell Biol.* 142 (1998) 523.
- [62] G. Solaini, D.A. Harris, *Biochem. J.* 390 (2005) 377.
- [63] J. Yang, P. Cron, V.M. Good, V. Thompson, B.A. Hemmings, D. Barford, *Nat. Struct. Biol.* 9 (2002) 940.
- [64] J. Yang, P. Cron, V. Thomson, V.M. Good, D. Hess, B.A. Hemmings, D. Barford, *Mol. Cell* 9 (2002) 1227.
- [65] M. Sukanuma, H. Fujiki, S. Okabe, S. Nishiwaki, D. Brautigan, T.S. Ingebritsen, M.R. Rosner, *Toxicon* 30 (1992) 873.
- [66] M. Roberge, C. Tudan, S.M.F. Hung, K.W. Harder, F.R. Jirik, H. Anderson, *Cancer Res.* 54 (1994) 6115.
- [67] S. Mitsuhashi, N. Matsuura, M. Ubukata, H. Oikawa, H. Shima, K. Kikuchi, *Biochem. Biophys. Res. Commun.* 287 (2001) 328.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49

Table 1.

Quantitative PCR primers. Nucleotide positions in transcripts are shown in parentheses for each primer. mRNA sequences for established genes were obtained from the Rat Genome Database (<http://rgb.mcw.edu>, viewed at <http://www.ncbi.nlm.nih.gov/entrez>).

Gene	Accession no./probe set	Size (bp)	Forward primer	Reverse primer
NDR1	NM_001015025	176	TTCGAGGGCCTGACAGCCAG (807-826)	TCAGAGACTTGACTAGGACGCAGT (960-983)
NDR2	EF444939.1	238	AGGTCATCCGTTCTTTGAGGGTGTG (1284-1308)	ACGTGGGGATGGAGCCTCGCTG (1501-1522)
Gapdh	NM_017008	83	GCTGGCATTGCTCTCAATGACA (1738-1759)	TCCACCACCCTGTTGCTGTA (1801-1820)

Table 2. Potencies of PPP1/PPP2 inhibitors.

Inhibitor	IC ₅₀ against		Reference
	PPP1	PPP2	
Calyculin A	0.3 nM	0.13 nM	[65]
Okadaic acid	3.4 nM	0.07 nM	[65]
Microcystin LR	0.1 nM	0.1 nM	[65]
Fostriecin	4 μM	40 nM	[66]
Tautomycetin	1.6 nM	62 nM	[67]

Figure legends

1
2
3
4 Fig. 1. Quantitative PCR of (A) NDR2 and (B) NDR1 mRNA abundances in cardiac
5 myocytes exposed to endothelin-1 (ET-1). Myocytes (4 independent preparations for NDR2,
6 5 independent preparations for NDR1) were exposed to 100 nM ET-1 for the times indicated
7 and RNA extracted. Transcript levels were normalised to those of Gapdh, then expressed
8 relative to the zero-time controls. Results are expressed as means \pm SEM. Statistical
9 significance *versus* the zero-time control: *, $P < 0.05$; **, $P < 0.01$ by a paired two-tailed
10 Student's *t* test.
11
12
13
14
15
16
17
18
19
20

21 Fig. 2. Phosphorylation of NDR1(Thr-444) and NDR2(Thr-442), and activation of FLAG-
22 NDR2 in cardiac myocytes exposed to calyculin A. All results are expressed as means \pm
23 SEM. (A) Myocytes (3 independent preparations) were exposed to 200 nM calyculin A for
24 the times indicated and phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) assessed by
25 immunoblotting with an anti-NDR phosphopeptide antibody. There were no changes in the
26 abundances of total NDR1/NDR2. The intensities of the NDR1(*P*Thr-444)/NDR2(*P*Thr-442)
27 signals were assessed by scanning densitometry and expressed relative to the maximum values
28 which occurred after either 45 or 90 min of exposure of myocytes to calyculin A, depending
29 on the time point at which the greater extent of phosphorylation was observed. (B) The
30 dependence of NDR1(Thr-444)/NDR2(Thr-442) phosphorylation on calyculin A
31 concentration was assessed in myocytes (4 independent preparations) exposed to calyculin A
32 for 20 min. The intensities of the NDR1(*P*Thr-444)/NDR2(*P*Thr-442) signals were assessed
33 as described under (A) and expressed relative to the maximum values which occurred at either
34 100 or 300 nM calyculin A, depending on the concentration at which the greater extent of
35 phosphorylation was observed. (C) Myocytes were infected with Adv.FLAG-NDR2.
36 Immunoblotting with an anti-NDR1/NDR2 antibody showed that endogenous NDR1/NDR2
37 and FLAG-NDR2 were expressed in approximately equal amounts. (D) The dependence of
38 FLAG-NDR2 activity on calyculin A concentrations was assessed in infected myocytes (4
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 independent preparations) exposed to calyculin A for 20 min. Activities were expressed
2 relative to the maximum values which occurred at either 100 or 300 nM calyculin A,
3
4 depending on the concentration at which the greater activity was observed.
5
6
7

8 Fig. 3. Phosphorylation of NDR1(Thr-444) and NDR2(Thr-442) in cardiac myocytes exposed
9 to hyperosmotic shock or oxidative stress. All results are expressed as means \pm SEM. (A and
10 B) Myocytes (3 independent preparations) were exposed to hyperosmotic shock (0.5 M
11 sorbitol) (A) or 0.5 mM H₂O₂ (B) and phosphorylation of NDR1(Thr-444)/NDR2(Thr-442)
12 assessed by immunoblotting with an anti-NDR phosphopeptide antibody. There were no
13 changes in the abundances of total NDR1/NDR2. The intensities of the NDR1(*P*Thr-
14 444)/NDR2(*P*Thr-442) signals were assessed by scanning densitometry and were expressed
15 relative to the maximum values which occurred after either 10 or 20 min of exposure of
16 myocytes to hyperosmotic shock (A) or 20 or 45 min of exposure to 0.5 mM H₂O₂ (B),
17 depending on the time point at which the greater extent of phosphorylation was observed. (C)
18 The dependence of NDR1(Thr-444)/NDR2(Thr-442) phosphorylation on H₂O₂ concentration
19 was assessed in myocytes (5 independent preparations) exposed to H₂O₂ for 20 min. The
20 intensities of the NDR1(*P*Thr-444)/NDR2(*P*Thr-442) signals were assessed as described
21 under (A and B) and were expressed relative to the maximum values which occurred at 0.5
22 mM H₂O₂.
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41

42 Fig. 4. Direct comparison of induction of phosphorylation of NDR1(Thr-444) and
43 NDR2(Thr-442), and activation of FLAG-NDR2 in cardiac myocytes exposed to a range of
44 interventions. HOS, hyperosmotic shock; OKA, okadaic acid. (A) Myocytes (3 to 8
45 independent preparations) were exposed to NDR modulators at the concentrations and for the
46 times indicated and phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) assessed by
47 immunoblotting with an anti-NDR phosphopeptide antibody. The intensities of the
48 NDR1(*P*Thr-444)/NDR2(*P*Thr-442) signals were assessed by scanning densitometry. Results
49 are expressed relative to maximum phosphorylation which was observed with 200 nM
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 calyculin A at either 20 min or 60 min, depending on the time point at which the greater
2 extent of phosphorylation was observed, and are means \pm SEM. Statistical significance: *,
3 $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$ versus control or †, $P < 0.01$ versus OKA (1 μ M, 60 min)
4 by a 1-tailed ANOVA with Tukey's multiple comparison test. (B and C) FLAG-NDR2
5 activity following exposure of myocytes [8 independent preparations in (B), 3 independent
6 preparations in (C)] to the interventions indicated and expressed relative to the maximum
7 activity which was observed with 200 nM calyculin A at either 20 min or 60 min, depending
8 on the time point at which the greater activity was observed. Results are means \pm SEM. (D)
9 Myocytes expressing the FLAG-NDR2 transgene (2 independent preparations of myocytes)
10 were exposed to NDR modulators at the concentrations and for the times indicated and
11 phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) assessed by immunoblotting with an
12 anti-NDR phosphopeptide antibody. The intensities of the NDR1(*P*Thr-444)/NDR2(*P*Thr-
13 442) signals were assessed as under (A). The upper band represents FLAG-NDR2(*P*Thr-442)
14 and the two lower bands represent NDR1(*P*Thr-444)/NDR2(*P*Thr-442). The unfilled bars in
15 the histogram represent FLAG-NDR2(*P*Thr-442) whereas the filled bars represent
16 endogenous NDR1(*P*Thr-444)/NDR2(*P*Thr-442). Results are expressed relative to maximum
17 phosphorylation which was observed with 200 nM calyculin A at 60 min and are means \pm SD.
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37

38 Fig. 5. Inhibition of calyculin A-induced activation of FLAG-NDR2 and phosphorylation of
39 endogenous NDR1(Thr-444)/NDR2(Thr-442) and FLAG-NDR2(Thr-442) by staurosporine.
40 Adv, adenoviral vector. (A and B) Myocytes were preincubated for 20 min with staurosporine
41 at the concentrations indicated, then were exposed to 50 nM calyculin A for a further 20 min.
42 (A) Inhibition of calyculin A-induced stimulation of FLAG-NDR2 activity by staurosporine.
43 FLAG-NDR2 activity in control 1 was assessed at 50 nM calyculin A (20 min) in the absence
44 of staurosporine and results were expressed relative to this. FLAG-NDR2 activity in control 2
45 was assessed in the absence of 50 nM calyculin A and staurosporine. (B) Inhibition of
46 calyculin A-induced phosphorylation of endogenous NDR1(Thr-444)/NDR2(Thr-442) (left
47 hand immunoblot panel and unfilled bars), and of endogenous NDR1(Thr-444)/NDR2(Thr-
48
49
50
51
52
53
54
55
56
57
58
59

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

442) and FLAG-NDR2(Thr-442) (right hand immunoblot panel and filled bars) was assessed by immunoblotting with an anti-NDR phosphopeptide antibody. Intensities of the endogenous NDR1(PThr-444)/NDR2(PThr-442) signals (left hand immunoblot panel) or endogenous NDR1(PThr-444)/NDR2(PThr-442) + FLAG-NDR2(PThr-442) (right hand immunoblot panel) were quantified by scanning densitometry and expressed relative to the values obtained with calyculin A (50 nM, 20 min) for each single experimental grouping. Results are means \pm SEM for 3 (A) or 4 (B) independent preparations of myocytes.

Fig. 6. Stimulation of NDR2 activity in the FLAG-NDR2-PIFtide chimera. Myocytes were infected with an adenovirus (Adv) empty vector, with Adv.FLAG-NDR2(wild-type) or with Adv.FLAG-NDR2-PIFtide. The relative levels of expression of FLAG-NDR2(wild-type) and FLAG-NDR2-PIFtide were assessed with an antibody to the FLAG epitope (upper panel). Myocytes expressing the transgenes were exposed to 200 nM calyculin A for 20 min and the activities of FLAG-NDR2(wild type) or FLAG-NDR2-PIFtide were measured (lower panel). Results are expressed relative to the FLAG-NDR2(wild-type) infected myocytes exposed to 200 nM calyculin A for 20 min and are means \pm SEM for 6 independent preparations of myocytes. The results shown were not corrected for expression levels and the Adv blank was not subtracted (but see the Results section for these calculations).

Fig. 7. Subcellular localisation of FLAG-NDR2. Myocytes were infected with Adv.FLAG-NDR2 and were stained with (A) monoclonal anti-FLAG, (B) phalloidin, or (C) Hoechst 33258 and a representative image of a single field is shown.

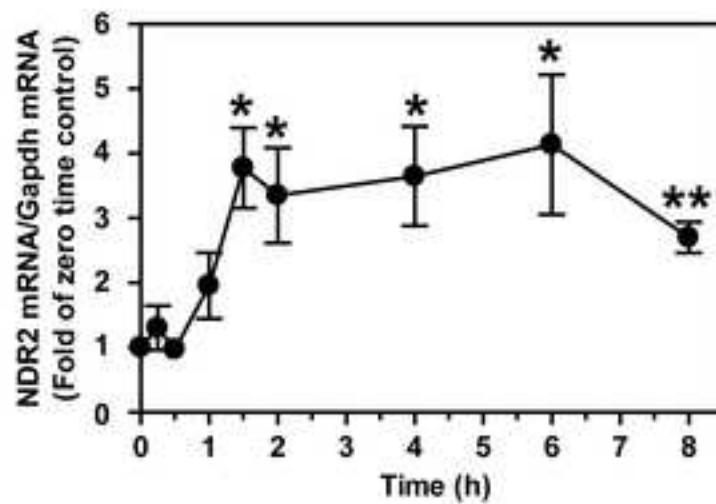
Fig. 8. Phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) in intact hearts. In each separate set of experiments, following a 15 min retrograde stabilisation perfusion, *ex vivo* rat heart preparations were either (i) perfused retrogradely for a further 20 min, (ii) rendered globally-ischemic for 10 min by occlusion of the aortic inflow, (iii) reperfused retrogradely for 10 min following global ischaemia (10 min), or (iv) perfused retrogradely with 40 nM calyculin A for

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

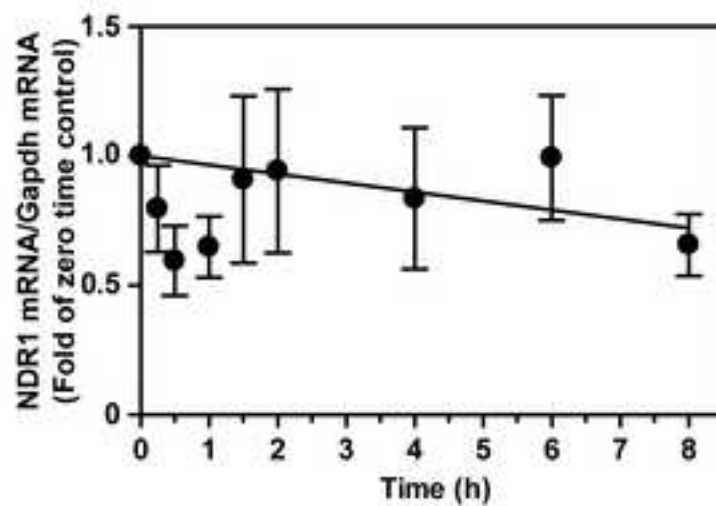
5 min. Phosphorylation of NDR1(Thr-444)/NDR2(Thr-442) was assessed by immunoblotting with an anti-NDR phosphopeptide antibody, and intensities of the NDR1(*P*Thr-444)/NDR2(*P*Thr-442) signals were quantified by scanning densitometry and expressed relative to the values obtained with perfusions with calyculin A (40 nM, 5 min) for each single experimental grouping. Results are means \pm SEM for 8 complete sets of experiments. Statistical significance *versus* the control perfusions: *, $P < 0.0001$ *versus* control by an unpaired two-tailed Student's *t* test.

Fuller et al. Fig. 1

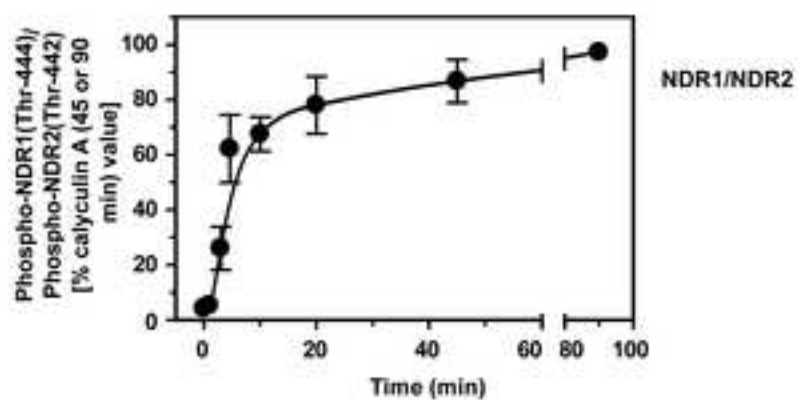
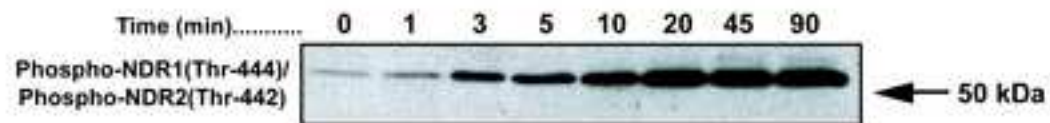
A



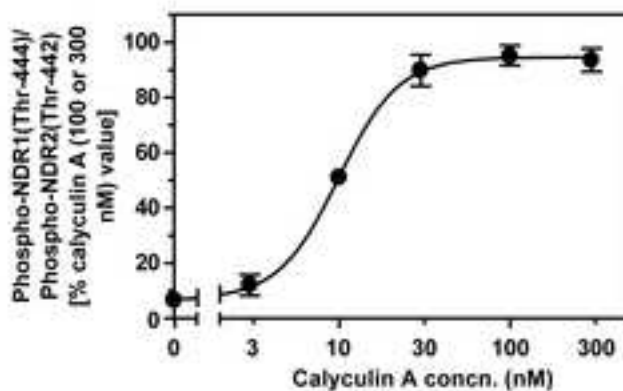
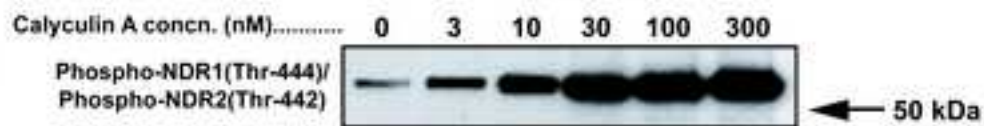
B



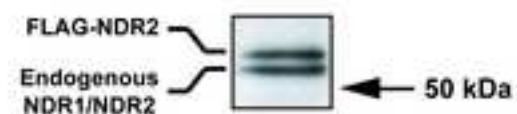
A



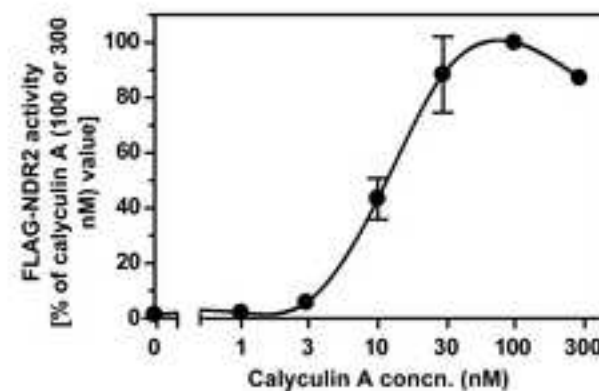
B



C

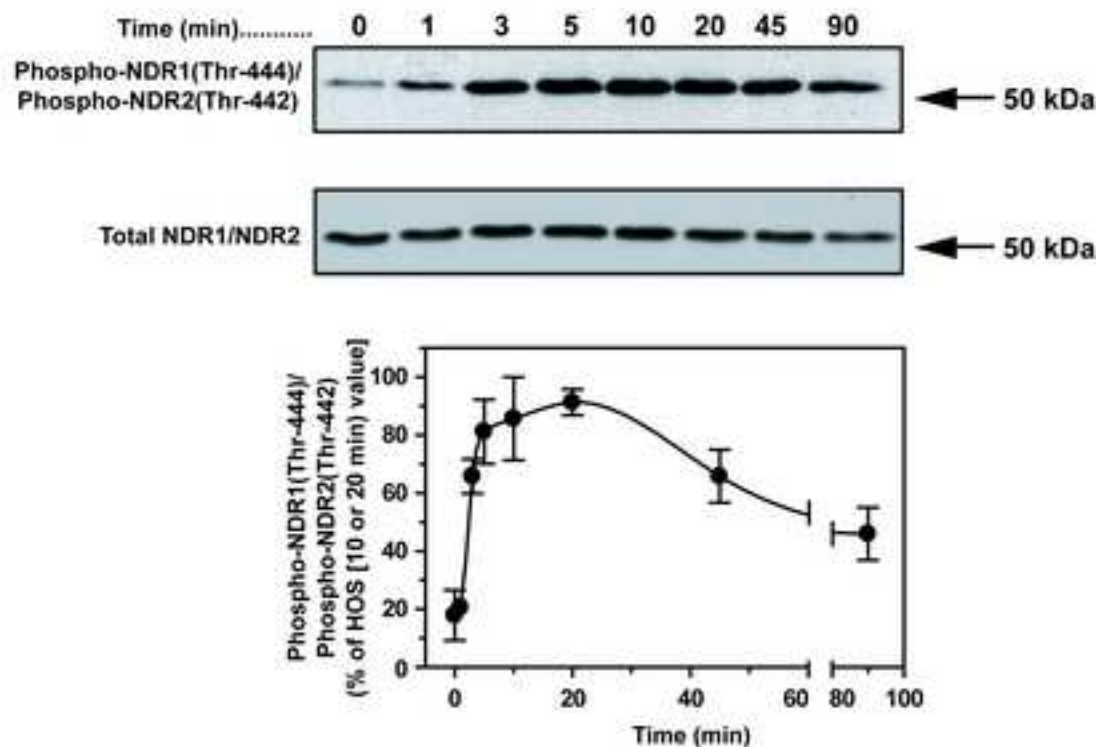


D

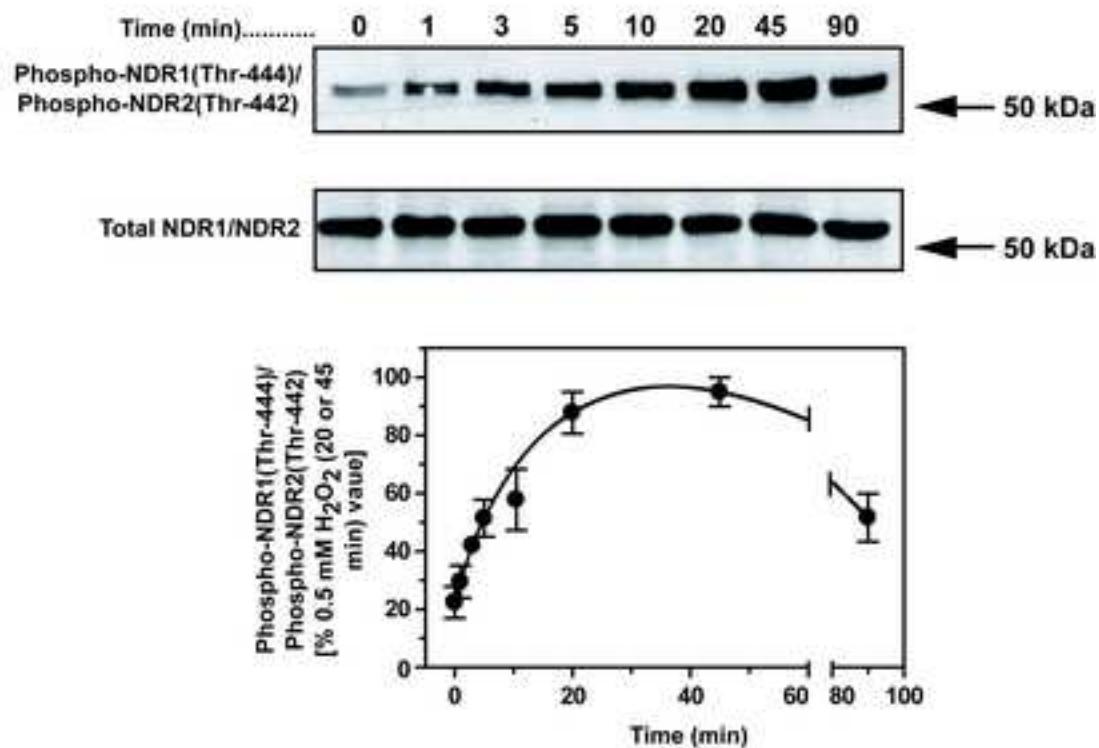


Fuller et al. Fig. 3

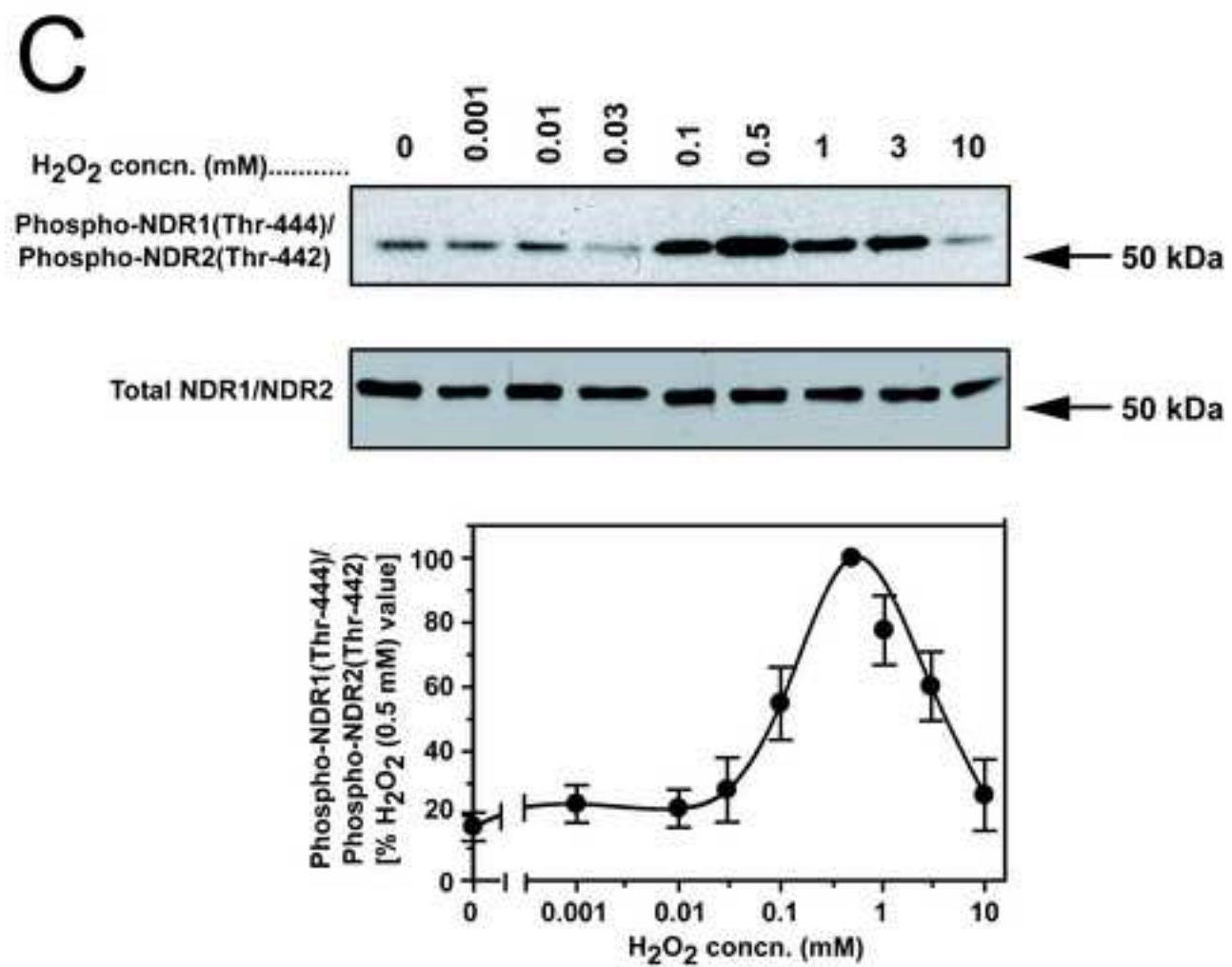
A



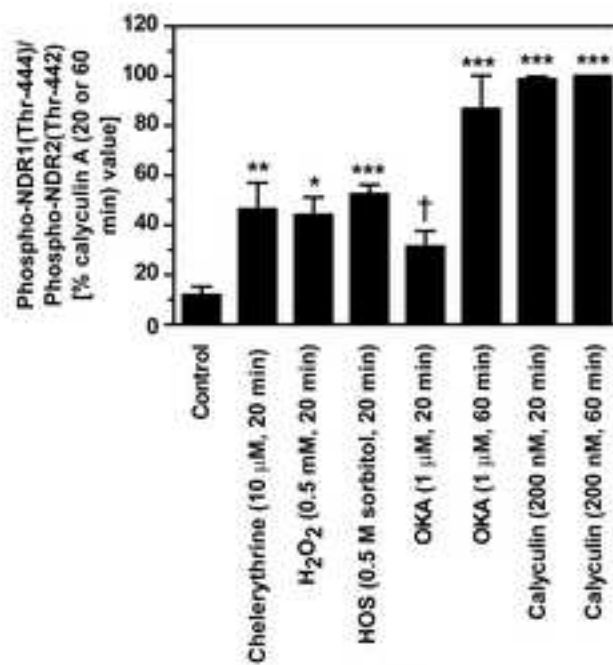
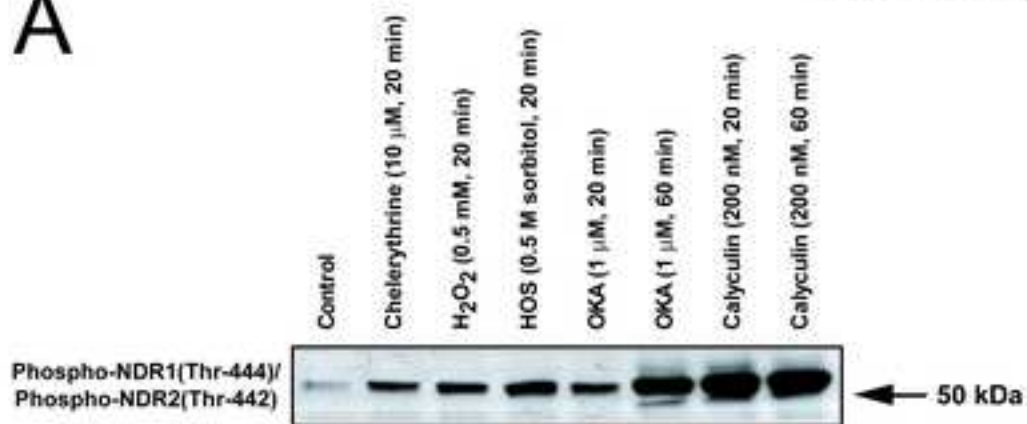
B



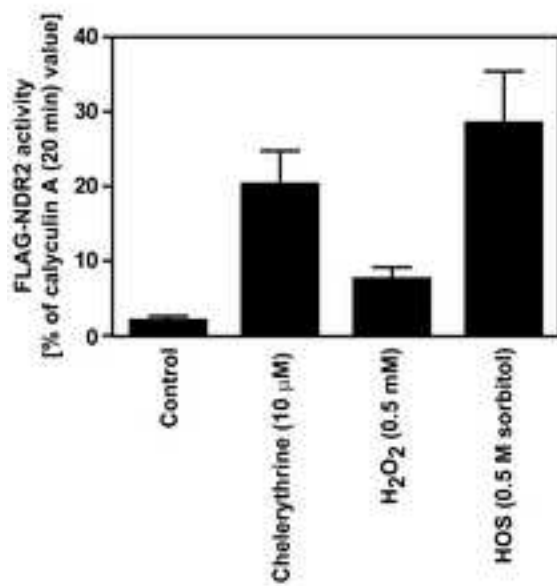
Fuller et al. Fig. 3



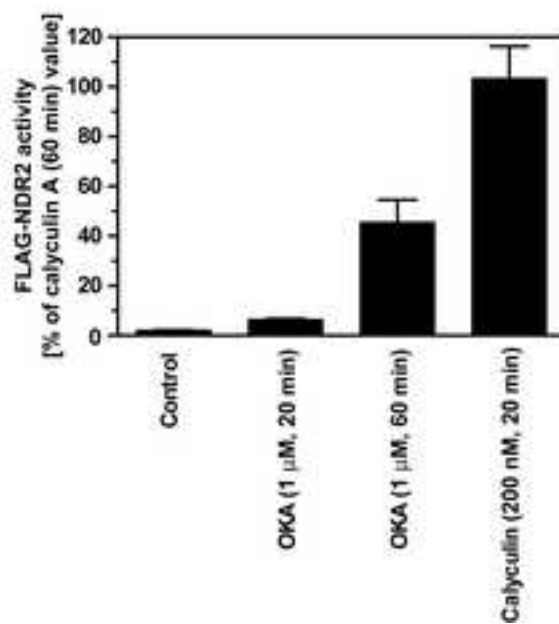
A



B

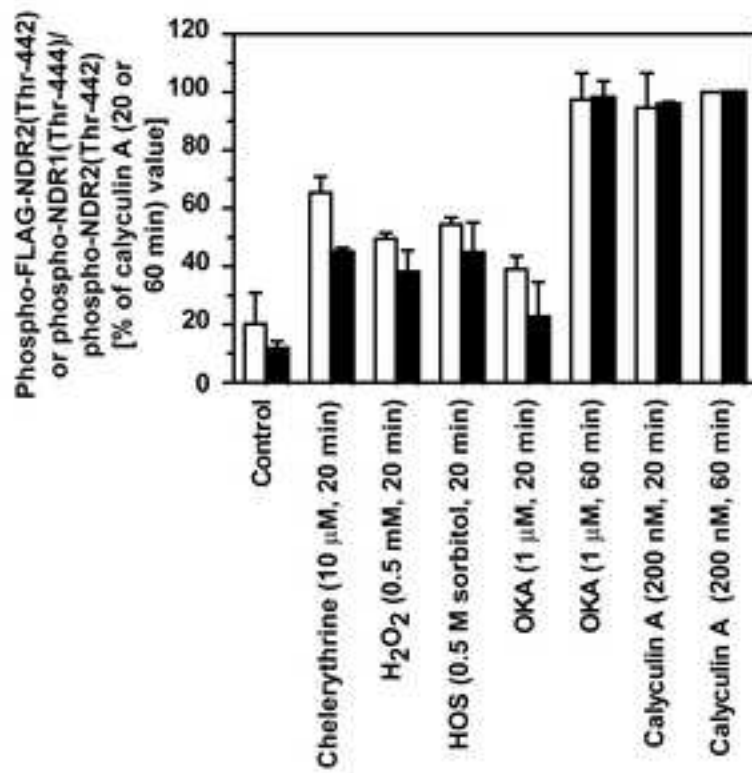
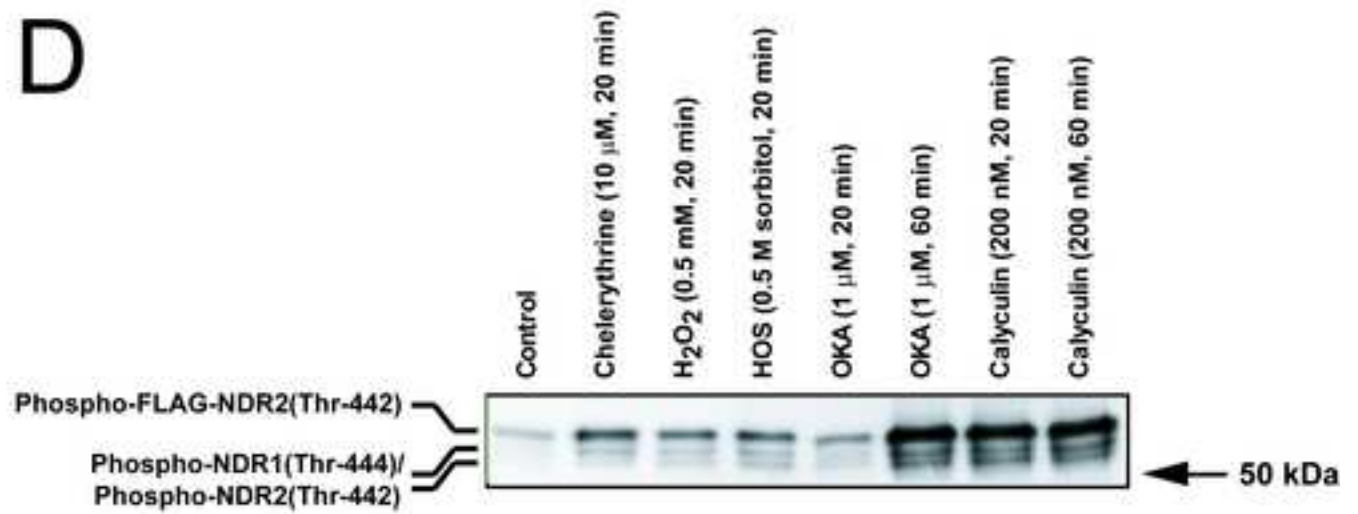


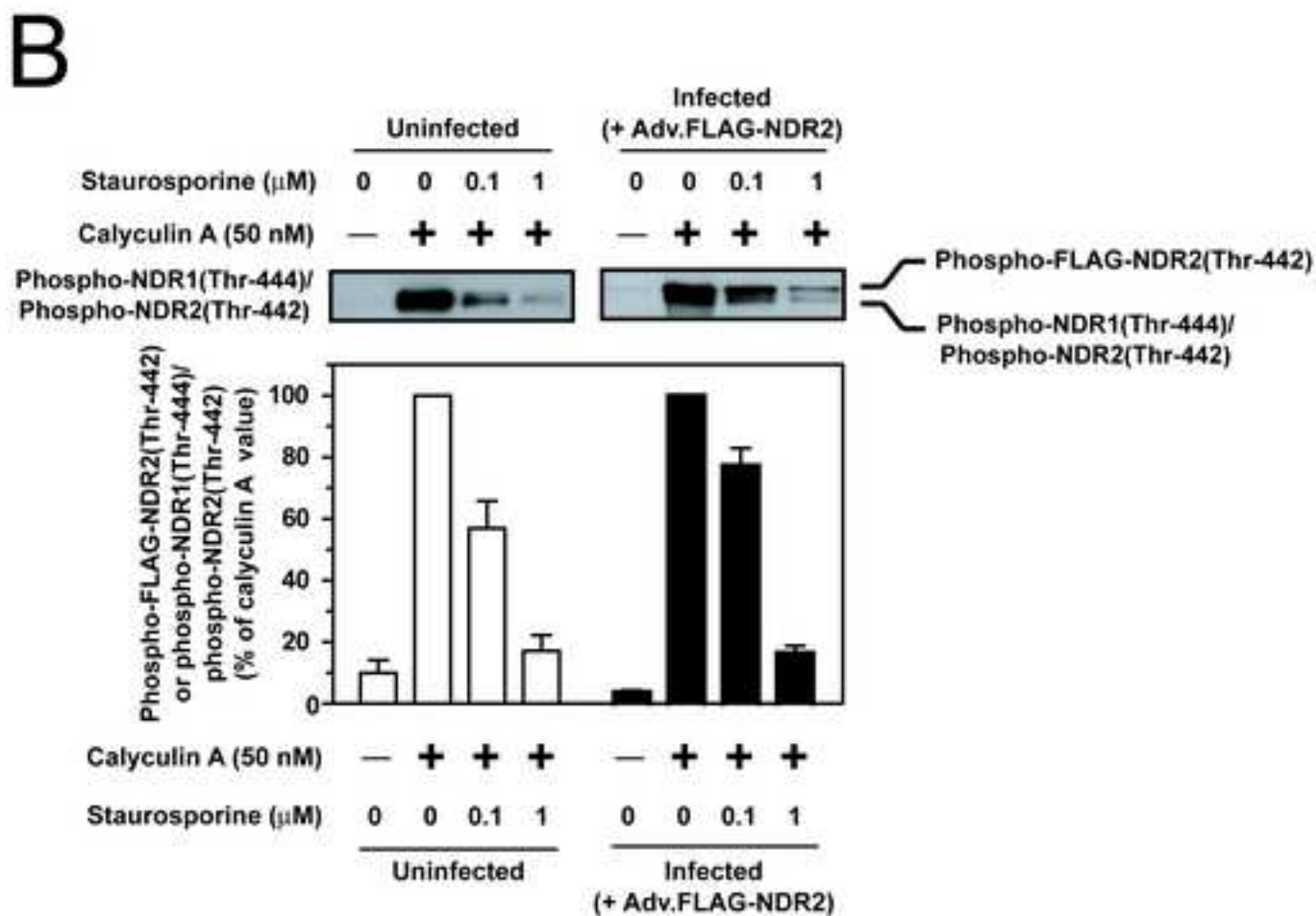
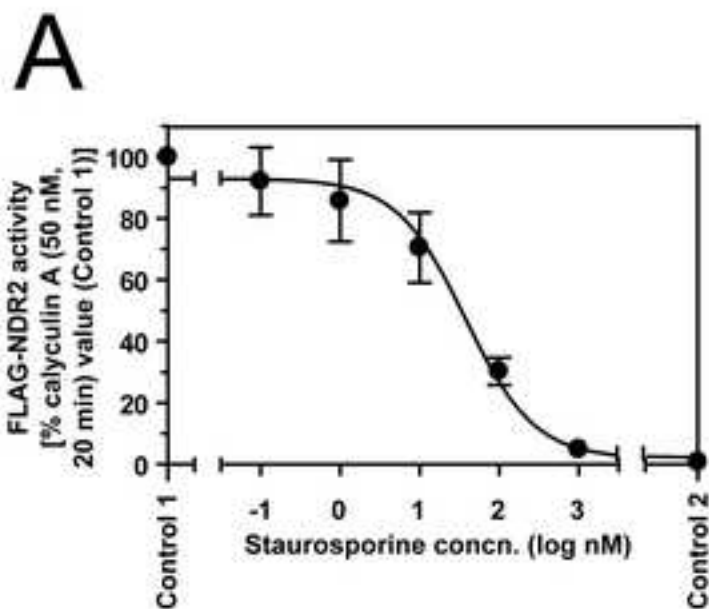
C



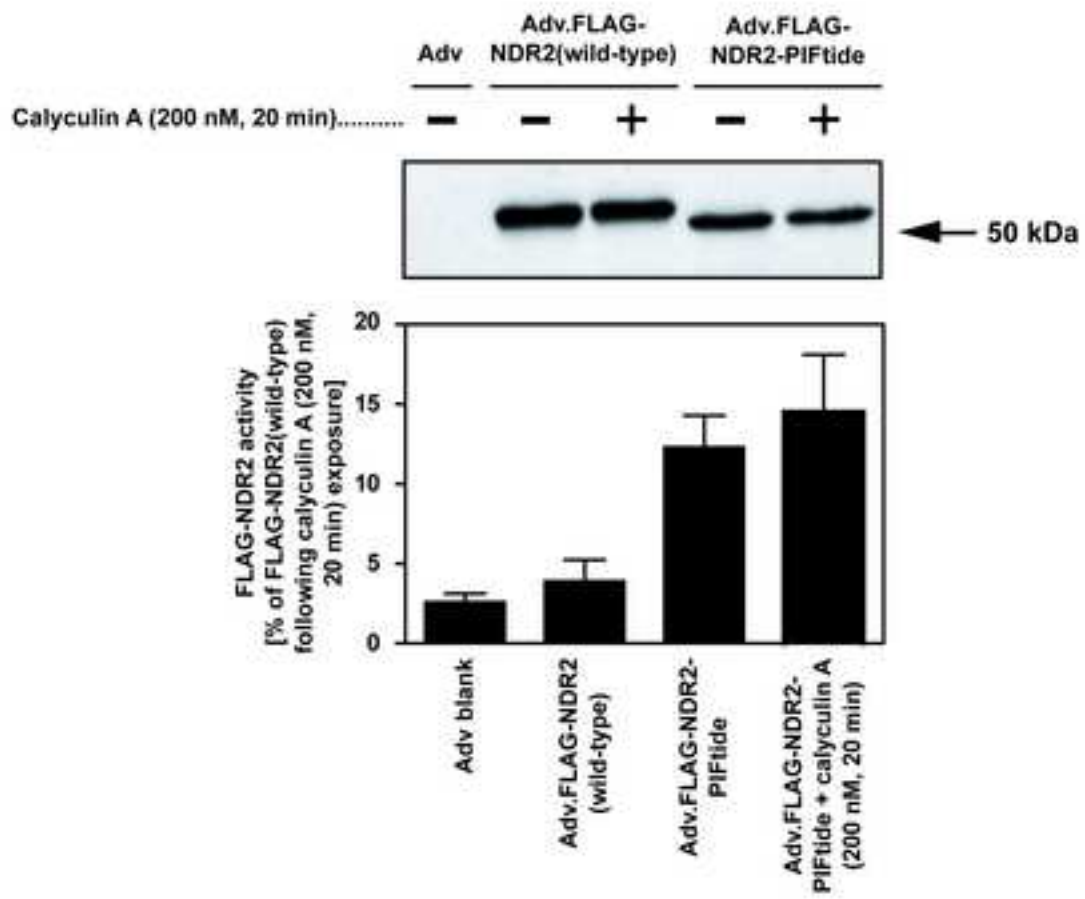
Fuller et al. Fig. 4

D

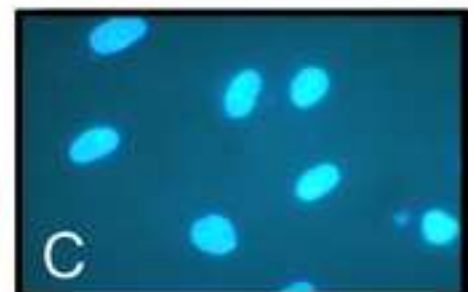
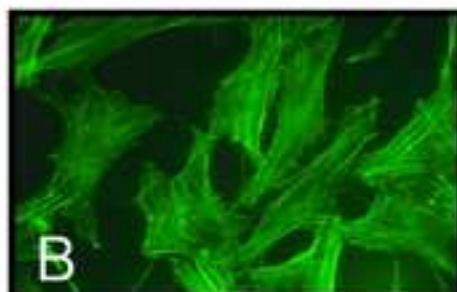




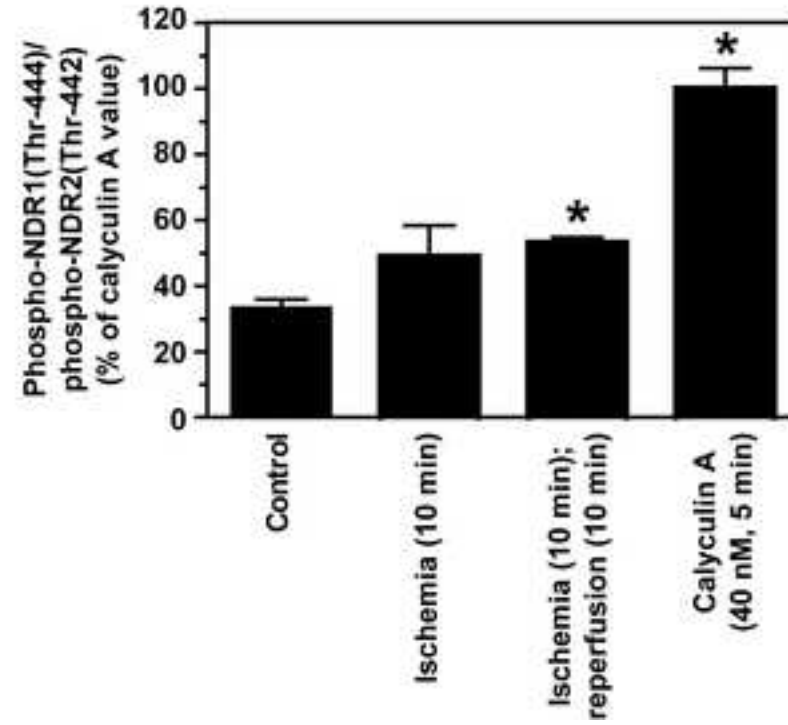
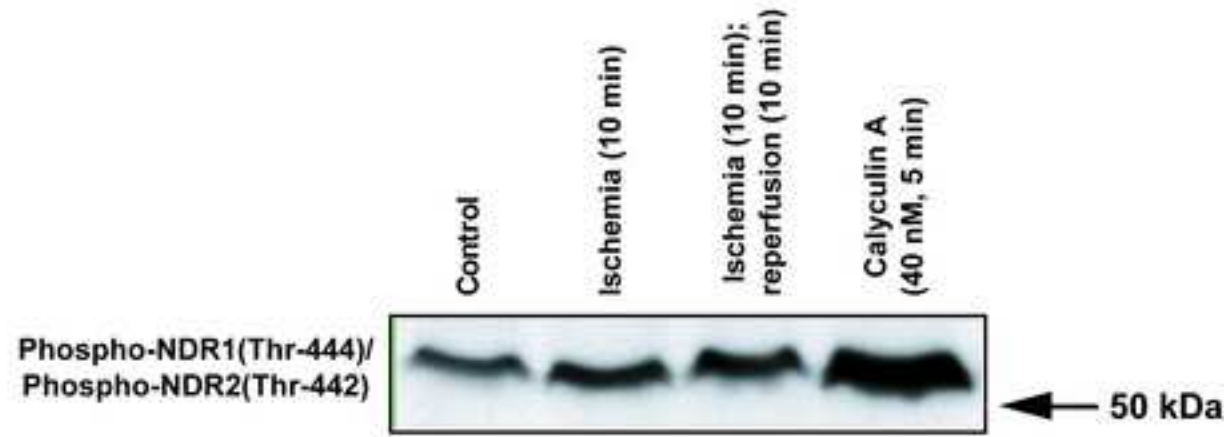
Fuller et al. Fig. 6



Fuller et al. Fig. 7



Fuller et al. Fig. 8



Supplementary material

Sequencing of rat NDR2 mRNA

Mouse NDR2 mRNA (Genbank accession no. NM_172734.2) was used to search the rat genome (www.ncbi.nlm.nih.gov/BLAST/) for the orthologous rat NDR2. Because of the incompleteness of the rat genomic sequence, only a partial sequence was identified on rat chromosome 4. Mouse NDR2 mRNA was then used to search for expressed sequence tags (ESTs) allocated to that region of rat chromosome 4 (Genbank accession nos. CV126295.1, CB720056.1, CB611889.1, CO568629.1, CV126546.1, BI290748.1 and CB801811.1). By combining sequences from the rat genome and these ESTs, the nucleotides comprising 1-1258 bp of the open reading frame of rat NDR2 mRNA were obtained. The last 336 bp of mouse NDR2 mRNA open reading frame were used to search against rat genome ESTs, and two additional ESTs were identified (Genbank accession nos. CV126295.1 and CB771034.1). Following this *in silico* approach, 1-1258bp and 1293-1592bp were obtained for rat NDR2 mRNA. cDNA prepared from neonatal rat ventricular myocytes was used as a template for PCR amplification with sets of primers (spanning predicted exon-exon boundaries) based on the *in silico* sequence (Supplementary Material, Table 1A). The resulting RT-PCR products were detected by SYBR green staining of agarose gel electrophoretograms and the bands captured under UV illumination. Bands were excised from the gel and purified using QIAQUICK spin columns. The sequence of PCR product with each primer pair was verified by automated sequencing with the indicated (Table 1B) primers (Advanced Biotechnology Centre, Imperial College London) using an ABI 3100 Genetic Analyser. PCR products were sequenced from both ends with the primers originally used for PCR.

Rapid amplification of cDNA ends (RACE) analysis

Total RNA was extracted using RNA-Bee from 3 separate myocyte preparations. Quantity and purity of RNAs were assessed from the A_{260} and the A_{260}/A_{280} ratio (1.8-2.0). Total RNA (150 : g) from control or endothelin-1 (ET-1, 100 nM, 2 h) treated myocytes was used for poly(A)⁺ RNA purification with oligo dT-cellulose (Biochemica, distributed by Sigma-Aldrich Company Ltd., U.K.). Briefly, total RNA dissolved in deionised water (600 : l) was incubated for 5 min at 65°C followed by addition of 1 vol. of 2 × Binding Buffer [1 M NaCl, 20 mM Tris pH 7.5, 2 mM EDTA, 0.1% (w/v) SDS]. Oligo dT-cellulose (5 mg) in 100 : 1 Binding Buffer was added and mixtures were rotated in room temperature for 15 min. The oligo dT-cellulose was washed twice with Binding Buffer (1 ml) and twice with Washing Buffer [1 ml; 0.2 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.05 % (w/v) SDS]. Poly(A)⁺ RNA was eluted from the oligo dT-cellulose by incubation (2 × 5 min, 37°C) in Elution Buffer [250 : l; 10 mM Tris pH 7.5, 1 mM EDTA, 0.05% (w/v) SDS]. The purification procedure was repeated and poly(A)⁺ RNA was precipitated with 4 M NaCl (50 : l) and two vol. absolute ethanol, followed by centrifugation (10,000 × g, 15 min, 4°C). After washing with 70% (v/v) ethanol and repeated centrifugation, poly(A)⁺ RNA was dissolved in deionised water (8 : l). Poly(A)⁺ RNA was reverse-transcribed using a SMART™ RACE cDNA Amplification Kit (Clontech). Poly(A)⁺ RNA (0.9 : g) was mixed with 10 mM oligo-dT primer (1 : 1, 5'-CDS primer A or 3'-CDS primer A), 10 mM SMART™ II A oligonucleotide (1 : 1), and deionised water to a total volume of 5 : l. The reaction mixture was heated (2 min, 72°C) and cooled on ice. Subsequently, 5 × Reaction Buffer (2 : l; 250 mM Tris-HCl, pH 8.3, 30 mM MgCl₂, 375 mM KCl), 10 mM dNTP (1 : 1), 20 mM dithiothreitol (DTT, 1 : 1) and PowerScript™ reverse transcriptase (1 : 1) were added and mixture was incubated at 42°C for 90 min. Reverse transcriptase was then inactivated

(72°C, 7 min). RACE cDNA reaction mixture (10 : 1) was diluted to 250 : 1 with Tricine-EDTA (10 mM Tricine pH 8.5, 1 mM EDTA) and stored at -80°C. Sequence corresponding to either 5'-end or 3'-end of NDR2 mRNA were amplified using SMART™ RACE cDNA Amplification Kit's Universal Primer A Mix (UPM, complementary for the extended 5'-end of the 5'-RACE cDNA or extended of 3'-end of 3'-RACE cDNA after the poly-A tail) and NDR2 gene specific primers. For each PCR reaction, 2.5 : 1 of RACE cDNA was combined with 10 × Advantage® 2 Polymerase Buffer [Clontech, 10 : 1; 40 mM Tricine-KOH pH 9.2, 15 mM KOAc, 3.5 mM Mg(OAc)₂], NDR2 gene specific primer (1 : 1; 10 : M), 10 × UPM (5 : 1), dNTP (10 mM) and Advantage® 2 Polymerase Mix (1 : 1). The polymerase chain reaction (PCR) program was as follows: 94°C, 5min; 5 cycles of (94°C, 30 s; 72°C, 2 min), 5 cycles of (94°C, 30 s; 70°C, 30 s; 72°C, 2 min), 25 cycles (94°C, 30 s; 68°C, 30 s; 72°C, 2 min) and 72°C, 4 min. The PCR reaction mixture was diluted 1:50 with MilliQ water and the diluted RACE reaction (2.5 : 1) was used as a template in nested PCR with appropriate NDR2 primers (Supplementary Material, Table 1C) and the SMART™ RACE cDNA Amplification Kit Nested UPM primer using a similar PCR protocol as in original RACE PCR. Nested PCR products were analysed on SYBR Green-stained agarose gels and the bands were captured under UV illumination. Products were excised from the gel and purified using QIAQUICK PCR purification columns for automated sequencing (Advanced Biotechnology Centre, Imperial College London) using an ABI 3100 Genetic Analyser. The ClustalW program (www.ebi.ac.uk/clustalw) was used for multiple alignment of sequencing results (Supplementary Material, Fig. 1), and the rat NDR2 mRNA sequence submitted to Genbank (accession no. EF444939.1).

Adenoviral vectors

Adenoviral vectors were prepared using the AdEasy™ XL Adenoviral Vector System (Stratagene). To facilitate identification, separation and immunoprecipitation of virally infected proteins from endogenous NDR2, adenoviral constructs were tagged with a FLAG® sequence (DYKDDDDK) at their N-terminus. A FLAG® sequence was first introduced between the BglII and KpnI restriction sites in the multiple cloning site (MCS) of the pShuttle-CMV vector by synthesis of an oligonucleotide cassette containing the FLAG sequence preceded by an ATG start codon. The complementary oligonucleotides used were:

Forward:

5'-GATCTACCATGG**ACTACAAAGACGATGACGACAAG**GGTACCGTCGACGC-3';

Reverse:

5'-GGCCGCGTCGACGGTACC**CTTGTCGTCATCGTCTTTGTAGTCCATGGTA**-3' with the FLAG® sequence underlined and emboldened and its antisense complement underlined and italicised. The oligonucleotides were annealed and phosphorylated, resulting in a cassette flanked by "cut" BglII and NotI sites, which was then inserted into the corresponding restriction sites in the multiple cloning site of pShuttle-CMV. This strategy, which replaces the KpnI-NotI fragment of pShuttle-CMV with an identical sequence in the cassette insert, was used to avoid the potential difficulty of cutting both the BglII and KpnI sites which lie immediately adjacent to each other in pShuttle-CMV.

The rat NDR2 coding sequence was subsequently amplified from rat cDNA by PCR with Pfu polymerase using forward primer

(5'-GACGACAAG**GGTACCAT**GGCAATGACGGCAGGGGC-3') and reverse primer

(5'-CTTATCTAG**AAGCTT**CATAACTTCCCAGCTTTC-3') to produce NDR2 flanked by

KpnI (forward primer) and HindIII (reverse primer) sites which are underlined and emboldened. These were then used for insertion into the corresponding sites in the prepared FLAG-pShuttle-CMV, downstream and in frame with the FLAG tag sequence. NDR2PIF was prepared similarly but using reverse primer 5'-CTTATCTAG**AAGCTT**TCACCAGTCGGCGATGTAGTCGAAGTCGCGGAACATCT CCTGCTCCTCTTTGTAGTCAGGCTCTGTGGTATTCG-3' which generates a product in which the C-terminal hydrophobic domain of NDR2 (residues 433-464) is replaced with the 3-phosphoinositide-dependent kinase 1 interacting fragment (PIF) of human protein kinase N2 (PKN2, NP_006247 residues 969-983, sequence EQEMFRDFDYVADW), as has been previously performed for human NDR2 [1]. The orthologous sequence in rat PKN2 is identical to human PKN2 except for the substitution of Ile- for Val-980. All constructs were sequenced using an ABI 3100 Genetic Analyser (Advanced Biotechnology Centre, Imperial College London). Shuttle plasmids containing verified sequences were then linearised with PmeI and used to transform BJ5183-AD-1 cells which were subsequently screened for homologous recombination. Adenoviral plasmids from positive recombinants were expanded in XL10Gold cells, linearised with PacI and used to transform HEK293 cells, as described in the AdEasy™ XL Adenoviral Vector System manual. Viruses were amplified through subsequent re-infection of HEK293 cells.

Reference

[1] M.R. Stegert, R. Tamaskovic, S. J. Bichsel, A. Hergovich, B. A. Hemmings, *J. Biol. Chem.* 279 (2004) 23806.

SUPPLEMENTARY MATERIAL TABLE 1. Primer sequences.

A. Sequences for RT-PCR of NDR2 mRNA

Primer	Sequence (5'→3')	Position in NDR2 (EF444939.1)	Primer pairing
F1	TGGCAATGACGGCAGGGGCAAC	152→173	F1R1, F1R2
R1	TCCTCGTCTGCCAGTCCTTC	316←336	F1R1
R2	TTCTCCTCGTCTGCCAGTCC	319←338	F1R2
F2	ACTGTGGCCAAGCTCACGCTG	214→234	F2R3
R3	TGGCATAGATGTGCCCTGTG	483←502	F2R3
F3	TGTGGCCAAGCTCACGCTGG	216→235	F3R4
R4	TCATGGCATAGATGTGCCCT	486←505	F3R4
F4	AGAAGGACTGGCAGACGAGG	315→334	F4R5
R5	CTGAGATGTAGAACTGCGTC	717←736	F4R5
F5,	CAGGTGGCTCATATCCGAGC	541→560	F5R6, F5R7, F5R8
R6	AGCCTAGCTGGTGGATTGCGTC	754←775	F5R6
F7	ACACCAGACTACATTGCTCCAGAGG	1006→1030	F5R7
R8	AAGTCCTTGGCTTTCTCAGAGACG	1197←1220	F5R8
F8	TGGCATTTCCTCCAGAGGTGC,	1175→1195	F8R9, F8R10
R9	TGGAGCCTCGCTGGGTCAGC	1494←1513	F8R9, F8R10
F9	AGGTCATCCGTTCTTTGAGGGTGTG	1284→1308	F9R10
R10	ACGTGGGGATGGAGCCTCGCTG	1501←1522	F8R10, F9R10

B. Primers for NDR2 sequencing

Primer	Sequence (5'→3')	Position in NDR2 (EF444939.1)
F14	AGGTCATCCGTTCTTTGAGGGTG	1284→1306
R15	AGGTTCTACAGGCAGCTTTCATGG	2379→2356
F15	TCCGGAGCATAGATGACACCTCA	1355→1377
R14	ACGCACTAGGTAGGTTCTACAGGCAG	2365←2390
R15	AGGTTCTACAGGCAGCTTTCATGG	2356→2379
F16	CACATCGCACTGATGGCACC	1896→1915

C. Primer sequences for NDR2 RACE

Primer	Sequence (5'→3')	Position in NDR2 (EF444939.1)	Function
F10	ACGCAATCCACCAGCTAGGCTTCATCCACCGGGA	755→788	3'-RACE
F11	AGACGCTGGCATTTCCTCCAGAGGTGCCCGT	1169→1199	3'-RACE, Nested PCR for F10 product
F12	GGTTTGAAGGGCTGACCCAGCGAGGCTCCATCC	1484→1516	3'-RACE, Nested PCR for F11 product
R11	TGGACCAGCCGCACCTCTCCGAAAGCTCCTC	443←473	5'-RACE, Nested PCR for R12 product
R12	TCCCGGTGGATGAAGCCTAGCTGGTGGATTGCGTC	754←788	5'-RACE, Nested PCR for R13 product
R13	AGACGGGCACCTCTGGAGGAAATGCCAGCGTC	1170←1201	5'-RACE

SUPPLEMENTARY MATERIAL TABLE 2. Agonists and interventions ineffective in causing phosphorylation of endogenous NDR1/NDR2 or activation of FLAG-NDR2.

A. Agonists and interventions tested which did not increase phosphorylation of NDR1(Thr-444)/NDR2(Thr-442).

Endothelin-1 (100 nM, vasoactive peptide, pro-hypertrophic agonist in cardiac myocytes)

Tumor necrosis factor α (10 ng/ml, pro-inflammatory cytokine)

Interleukin 1 β (10 ng/ml, pro-inflammatory cytokine)

Leukemia inhibitory factor (10 nU/ml, gp130-linked cytokine receptor agonist)

Cardiotrophin-1 (1 nM, gp130-linked cytokine receptor agonist, pro-hypertrophic agonist in cardiac myocytes)

Ionomycin (1 : M)

A23187 (20 : M, Ca²⁺ ionophore)

Anisomycin (50 ng/ml, protein synthesis inhibitor, cytotoxic stressor)

Cycloheximide (20 : M, protein synthesis inhibitor, cytotoxic stressor)

Staurosporine (1 : M, generalized Ser-/Thr-protein kinase, pro-apoptotic in cardiac myocytes)

Cyclosporin A [1 : M, phosphoprotein phosphatase 3 (calcineurin) inhibitor]

Doxorubicin (5 : M, antineoplastic anthracycline, pro-apoptotic in cardiac myocytes and cardiomyopathic)

Insulin (300 nM, receptor protein tyrosine kinase agonist polypeptide)

Platelet-derived growth factor (20 ng/ml, receptor protein tyrosine kinase agonist polypeptide)

Epidermal growth factor (20 ng/ml, receptor protein tyrosine kinase agonist polypeptide)

Phorbol 12-myristate 13-acetate (1 : M, activator of classical and novel isoforms of protein kinase C, pro-hypertrophic agonist in cardiac myocytes)

Phenylephrine (100 : M, α -adrenergic receptor agonist, pro-hypertrophic agonist in cardiac myocytes)

B. Agonists and agents tested which did not increase the activity of FLAG-tagged NDR2.

Na arsenite (0.5 mM, cytotoxic stressor)

Cycloheximide (20 : M, protein synthesis inhibitor, cytotoxic stressor)

Isoproterenol (10 : M, β -adrenergic receptor agonist, positive inotrope, lusitrope and chronotrope)

Phenylephrine (10 : M, α -adrenergic receptor agonist, pro-hypertrophic agonist in cardiac myocytes)

Norepinephrine (10 : M, α - β -adrenergic receptor agonist, pro-hypertrophic agonist in cardiac myocytes)

Endothelin-1 (100 nM, vasoactive peptide, pro-hypertrophic agonist in cardiac myocytes)

KCl (40 mM, cardioplegic intervention)

pH6.6 or pH 7.8 (pH lowered from or raised above control value of 7.4)

Lipopolysaccharides from *Escherichia coli* 0111.B4 or from *Salmonella typhimurium* serotype minnesota (500 ng/ml, endotoxin/cytotoxic stressors)

Lipopolysaccharides (rough strains) from *Salmonella typhimurium* serotype minnesota Re 595 (500 ng/ml, endotoxin/cytotoxic stressor)

SUPPLEMENTARY MATERIAL FIG. 1. Alignments are shown for the amino acid sequences encoded by the rat NDR2 (rNDR2, EF444939.1) cDNA, human NDR1 (hNDR1, NP_009202.1), mouse NDR1 (mNDR1, NP_598876.1), the predicted sequence for rNDR1 (genomic contig Rn20_WGA2119_4), hNDR2 (NP_055815.1) and mNDR2 (NP_766322.1). The peptide sequences to which the hNDR1 antibody was raised (59-86) and to which the hNDR1(*P*Thr-444) antibody was raised (436-450) are emboldened and underlined.

rNDR2	MAMTAGATTT	FPMSSTRER	VTVAKLTLEN	FYSNLILQHE	ERETRQKKE	VAMEEEGLAD	60
mNDR2	MAMTAGATTT	FPMSNHTRER	VTVAKLTLEN	FYSNLILQHE	ERETRQKKE	VAMEEEGLAD	60
hNDR2	MAMTAGTTTT	FPMSNHTRER	VTVAKLTLEN	FYSNLILQHE	ERETRQKKE	VAMEEEGLAD	60
rNDR1	MAMT-GSTPC	SSMSNHTKER	VTMTKVTLEN	FYSNLIAQHE	EREMRQKKE	KVMEEEGLKD	59
mNDR1	MAMT-GSTPC	SSMSNHTKER	VTMTKVTLEN	FYSNLIAQHE	EREMRQKKE	KVMEEEGLKD	59
hNDR1	MAMT-GSTPC	SSMSNHTKER	VTMTKVTLEN	FYSNLIAQHE	EREMRQKKE	KVMEEEGLKD	59
	****	.*.	.**.*:**	**:*:*:**	***** **	*** *****	.***** *
rNDR2	EKKLRRSQH	ARKETEFRL	KRTRLGLDDF	ESLKVIGRGA	FGEVRLVQKK	DTGHIYAMKI	120
mNDR2	EKKLRRSQH	ARKETEFRL	KRTRLGLDDF	ESLKVIGRGA	FGEVRLVQKK	DTGHIYAMKI	120
hNDR2	EKKLRRSQH	ARKETEFRL	KRTRLGLDDF	ESLKVIGRGA	FGEVRLVQKK	DTGHIYAMKI	120
rNDR1	EKKLRRSAH	ARKETEFRL	KRTRLGLEDF	ESLKVIGRGA	FGEVRLVQKK	DTGHVYAMKI	119
mNDR1	EKKLRRSAH	ARKETEFRL	KRTRLGLEDF	ESLKVIGRGA	FGEVRLVQKK	DTGHVYAMKI	119
hNDR1	EKKLRRSAH	ARKETEFRL	KRTRLGLEDF	ESLKVIGRGA	FGEVRLVQKK	DTGHVYAMKI	119
	:** *	*****	*****:**	*****	*****	***:*****	
rNDR2	LRKADMLEKE	QVAHIRAERD	ILVEADGAWV	VKMFYSFQDK	RNLYLIMEFL	PGDMMTLLM	180
mNDR2	LRKADMLEKE	QVAHIRAERD	ILVEADGAWV	VKMFYSFQDK	RNLYLIMEFL	PGDMMTLLM	180
hNDR2	LRKADMLEKE	QVAHIRAERD	ILVEADGAWV	VKMFYSFQDK	RNLYLIMEFL	PGDMMTLLM	180
rNDR1	LRKADMLEKE	QVGHIRAERD	ILVEADSLWV	VRMFYSFQDK	LNLYLIMEFL	PGDMMTLLM	179
mNDR1	LRKADMLEKE	QVGHIRAERD	ILVEADSLWV	VKMFYSFQDK	LNLYLIMEFL	PGDMMTLLM	179
hNDR1	LRKADMLEKE	QVGHIRAERD	ILVEADSLWV	VKMFYSFQDK	LNLYLIMEFL	PGDMMTLLM	179
	:**	**.******	*****. **	*****	*****	*****	
rNDR2	KKDTLTEEET	QFYISETVLA	IDAIHQLGFI	HRDIKPDNLL	LDAKGHVKLS	DFGLCTGLKK	240
mNDR2	KKDTLTEEET	QFYISETVLA	IDAIHQLGFI	HRDVKPDNLL	LDAKGHVKLS	DFGLCTGLKK	240
hNDR2	KKDTLTEEET	QFYISETVLA	IDAIHQLGFI	HRDIKPDNLL	LDAKGHVKLS	DFGLCTGLKK	240
rNDR1	KKDTLTEEET	QFYIAETVLA	IDSIHQLGFI	HRDIKPDNLL	LDSKGHVKLS	DFGLCTGLKK	239
mNDR1	KKDTLTEEET	QFYIAETVLA	IDSIHQLGFI	HRDIKPDNLL	LDSKGHVKLS	DFGLCTGLKK	239
hNDR1	KKDTLTEEET	QFYIAETVLA	IDSIHQLGFI	HRDIKPDNLL	LDSKGHVKLS	DFGLCTGLKK	239
	*****	***:*****	**:******	**:******	**:******	*****	
rNDR2	AHRTEFYRNL	THNPPSDFS	QNMNSKRKAE	TWKKNRRQLA	YSTVGTDPYI	APEVFMQTGY	300
mNDR2	AHRTEFYRNL	THNPPSDFS	QNMNSKRKAE	TWKKNRRQLA	YSTVGTDPYI	APEVFMQTGY	300
hNDR2	AHRTEFYRNL	THNPPSDFS	QNMNSKRKAE	TWKKNRRQLA	YSTVGTDPYI	APEVFMQTGY	300
rNDR1	AHRTEFYRNL	NHSLPSDFTF	QNMNSKRKAE	TWKRNRRLA	FSTVGTDPYI	APEVFMQTGY	299
mNDR1	AHRTEFYRNL	NHSLPSDFTF	QNMNSKRKAE	TWKRNRRLA	FSTVGTDPYI	APEVFMQTGY	299
hNDR1	AHRTEFYRNL	NHSLPSDFTF	QNMNSKRKAE	TWKRNRRLA	FSTVGTDPYI	APEVFMQTGY	299
	*****	.*.***:**	*****	***:*****	.*****	*****	
rNDR2	NKLCDWWSLG	VIMYEMLIGF	PPFCSETPQE	TYRKVMSWKE	TLAFPPEVPV	SEKAKDLILR	360
mNDR2	NKLCDWWSLG	VIMYEMLIGF	PPFCSETPQE	TYRKVMSWKE	TLAFPPEVPV	SEKAKDLILR	360
hNDR2	NKLCDWWSLG	VIMYEMLIGY	PPFCSETPQE	TYRKVMNWKE	TLVFPPEVPI	SEKAKDLILR	360
rNDR1	NKLCDWWSLG	VIMYEMLIGY	PPFCSETPQE	TYKKVMNWKE	TLTFPPEVPV	SEKAKGLILR	359
mNDR1	NKLCDWWSLG	VIMYEMLIGY	PPFCSETPQE	TYKKVMNWKE	TLTFPPEVPV	SEKAKGLILR	359
hNDR1	NKLCDWWSLG	VIMYEMLIGY	PPFCSETPQE	TYKKVMNWKE	TLTFPPEVPI	SEKAKDLILR	359
	*****	*****:	*****	**:***.***	**.******:	*****.***	
rNDR2	FCTDSENRI	NGGVEEIKGH	PFPEGVDWGH	IRERPAAIPI	EIRSIDDTSN	FDDFPESDIL	420
mNDR2	FCTDSENRI	NGGVEEIKGH	PFPEGVDWGH	IRERPAAIPI	EIRSIDDTSN	FDDFPESDIL	420
hNDR2	FCIDSENRI	NSGVEEIKGH	PFPEGVDWEH	IRERPAAIPI	EIKSIDDTSN	FDDFPESDIL	420
rNDR1	FCCEWEHRIG	APGVEEIKSN	PFPEGVDWEH	IRERPAAISI	EIKSIDDTSN	FDEFPESDIL	419
mNDR1	FCCEWEHRIG	APGVEEIKSN	PFPEGVDWEH	IRERPAAISI	EIKSIDDTSN	FDEFPESDIL	419
hNDR1	FCCEWEHRIG	APGVEEIKSN	SFFEGVDWEH	IRERPAAISI	EIKSIDDTSN	FDEFPESDIL	419
	** : *:**	*****:	***** *	*****.*	**:******	**.******	
rNDR2	QPVPNTT---	EPDYKSKDWV	FLNYTYKRFE	GLTQRGSIPT	YMKAGKL	464	
mNDR2	QPVPNTT---	EPDYKSKDWV	FLNYTYKRFE	GLTQRGSIPT	YMKAGKL	464	
hNDR2	QPVPNTT---	EPDYKSKDWV	FLNYTYKRFE	GLTQRGSIPT	YMKAGKL	464	
rNDR1	KPTVTTSNHP	ETDYKSKDWV	FINYTYKRFE	GLTARGAIPS	YMKAAK	465	
mNDR1	KPTVTTSSHP	ETDYKSKDWV	FINYTYKRFE	GLTARGAIPS	YMKAAK	465	
hNDR1	KPTVATSNHP	ETDYKSKDWV	FINYTYKRFE	GLTARGAIPS	YMKAAK	465	
	. :	*.***.***	*:*****	*** **:**	****.*:		